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ENZYMATIC PRODUCTION AND BIOLOGICAL ACTIVITIES OF CELLOBIO-OLIGOSACCHARIDES FROM LIGNOCELLULOSE

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

The Department of Food Science

by
Misook Kim
B.S., Dankook University, 2001
M.S., Dankook University, 2003
May 2008
DEDICATION

This work is dedicated to my parents, Yoontai Kim and Baekmo Jeong; my sister, Yeonsook Kim; my brother, Boyoung Kim; my brother in law, Wontaek Shin; and my niece, Yeji Shin for their love, endless support, and encouragement.
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Cellulose is the most abundant biopolymer in nature and is the major component of lignocellulosic biomass. It has potential to produce not only glucose but value-added products such as cellobiose and oligosaccharides. Typically, enzymatic hydrolysis of cellulose produces glucose and a small amount of cellobiose. Modification of cellulase solutions altered ratio of glucose to cellobiose produced. The addition of glucose oxidase, gluconolactone, or gluconic acid significantly increased the amount of cellobiose remaining at the end of cellulose hydrolysis. Addition of glucose oxidase resulted in cellobiose being 23.7% of the product sugars from pure cellulose and 14.1% from lignocellulose (sugarcane bagasse). The presence of gluconolactone in the reaction mixture increased cellobiose to 31.3% of the sugars from cellulose and 15.8% from bagasse. The presence of gluconic acid changed the cellobiose to 21.9% of the sugars from cellulose and 13.2% from bagasse. In order to produce cellbio-oligosaccharides, a dextranucrase from *Leuconostoc mesenteroides* B-512FMCM was employed to catalyze the transglycosylation reaction between cellobiose and sucrose. The major cellbio-oligosaccharides were the trisaccharides, α-D-glucopyranosyl-(1→2)-cellobiose and α-D-glucopyranosyl-(1→6)-cellobiose. Cellbio-oligosaccharides have valuable functional properties as potential antifungal and anticariogenic agents, and an α-glucosidase inhibitor.
CHAPTER 1. CELLOBIOSE PRODUCTION FROM CELLULOSE USING A CELLULASE MODIFICATION SYSTEM

*Portions of this chapter are reprinted by permission of Internaional Sugar Journal.
1.1. INTRODUCTION

Cellobiose is a homoglucan disaccharide linked β-1,4 and is a repeating unit of cellulose, the most abundant biopolymer in nature. It has enormous biotechnological potential as a source of renewable products in food, cosmetic, and pharmaceutical industries. Enzymes, capable of breaking down cellobiose, are absent in humans. It is also poorly fermented by intestinal microbes, having an estimated a caloric value of 2 kcal/g (Nakamura, 2004). Therefore, cellobiose in its own right can be an effective sugar for diabetes and obesity control if it can be produced economically. The structural property due to the β-1,4 linkages gives the potential to synthesize novel compounds with specific functionalities by using glucosyl transferase analogous to the α-linked glucosides now available (Cote, 2007). Cellobiose-acarbose analogues, produced by transglycosylation reactions are inhibitors of β-glucosidase, whereas acarbose is not (Lee et al., 2001; Morales et al., 2001). Also, the fully or partially esterified cellobiose is a strong agent or thickener for water-immiscible liquid carriers and sustains its own shape with rigidity in cosmetic uses (Franklin et al., 2001).

There is a lack of information on the economic cost and efficient production of cellobiose. Even though the oldest and easiest way to produce cellobiose is by acidic hydrolysis of cellulose, this process causes numerous problems including acid corrosion, neutralization requirements and side products causing difficulties in size control. Many of these problems can be avoided by using induced enzymatic hydrolysis of cellulose, which utilizes specific substrates under mild conditions, i.e. pH 4-7 and temperatures between 30-50°C (Homma et al., 1993). Cellulase, which hydrolyzes cellulose and consists of endo-β-glucanase (EC 3.2.1.4), exo-β-glucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) has been employed in this process (Henrissat et al., 1998). Cellobiose is released synergistically by endo- and exo-glucanase, and β-glucosidases. Cellobiose is then hydrolyzed to glucose by β-glucosidase. This enzyme is a potential target to control the product profile. Homma et al. (1993) removed β-glucosidase
from cellulase “complex” and produced cellobiose. However, they had to employ several processes, including affinity precipitation, pH control, and fractionation by gel chromatography to obtain a cellulase free of β-glucosidase. Tanaka and Oi (1985) produced 21 mg of cellobiose by polymerizing 30 g of glucose using a β-glucosidase with a three-day incubation.

Oxidized products of glucose, gluconolactone and gluconic acid, are known inhibitors of the cellulase system. Gluconolactone selectively inhibits the β-glucosidase activity in the cellulase system which includes endo-β-glucanase, exo-β-glucanase, and β-glucosidase (Holtzapple et al., 1990).

1.2. LITERATURE REVIEW

1.2.1. Cellulose

Cellulose constitutes the most abundant, renewable polymer resource in nature and is found primarily in plant cell walls. Except for cotton, present in a nearly pure state, cellulose fibers are embedded in a matrix with other structural polymers, primarily hemicellulose and lignin (Table 1.1). This limits the rate and extent of utilization of whole cellulose. Nevertheless, the use of cellulose as a precursor for the production of bio-ethanol and chemical production has been extensively studied because of its relatively low cost and plentiful supply (Lynd et al., 2002; Sun and Cheng, 2002).

Cellulose is composed of linear chains of β-(1→4)-D-glucopyranosyl units. Hydroxyl groups-OH at the C-2, the C-3, and the C-6 positions within an anhydroglucose unit exhibit different polarities contributing to the formation of various inter- and intramolecular hydrogen bonds. All the hydroxyl groups are bonded equatorially in the glucopyranose ring, which produce hydrophilic sites parallel to the ring plane. The CH groups are bonded in the glucopyranose ring axially, which causes a hydrophobic site perpendicular to the ring (Kondo, 2005). Within the elementary fibrils of cellulose the arrangement of individual chains has been inferred from fitting of X-ray diffraction data to statistical models, calculating structure based on minimum conformational energy. Approximately 30 individual cellulose molecules assemble into larger units known as protofibrils, which are then packed into larger units.
Table 1.1. Gross composition of some typical cellulose containing materials\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th>Source</th>
<th>Cellulose</th>
<th>Hemicellulose</th>
<th>Lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bagasse</td>
<td>40</td>
<td>24-30</td>
<td>20-25</td>
</tr>
<tr>
<td>Cotton</td>
<td>95</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Flax (retted)</td>
<td>71</td>
<td>21</td>
<td>2</td>
</tr>
<tr>
<td>Hardwood stems</td>
<td>40-50</td>
<td>24-40</td>
<td>18-25</td>
</tr>
<tr>
<td>Softwood stems</td>
<td>45-50</td>
<td>25-35</td>
<td>25-35</td>
</tr>
<tr>
<td>Corn cobs</td>
<td>45</td>
<td>35</td>
<td>15</td>
</tr>
<tr>
<td>Grasses</td>
<td>25-40</td>
<td>35-50</td>
<td>10-30</td>
</tr>
<tr>
<td>Paper</td>
<td>85-99</td>
<td>0</td>
<td>0-15</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>30</td>
<td>50</td>
<td>15</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Hon, 1996; \textsuperscript{b}Sun and Cheng, 2002.
called microfibrils, and these are in turn assembled into the familiar cellulose fibers. Cellulose microfibrils have regions of crystalline structure and both hydrophilic and hydrophobic regions due to the equatorially bonded HO-groups and axially bonded HC-groups (Sundari et al., 1991; Figure 1.1). Cellulose chains form numerous intra- and intermolecular hydrogen bonds, accounting for the formation of rigid and insoluble microfibrils. Adjacent sheets overlie one another and are held together by weak intersheet van der Waals forces. Strong inter- and intramolecular O-H···O bonds allow chains to co-crystallize shortly after biosynthesis. Highly crystalline microfibrils held together by hydrogen bonds, hydrophobic interactions and van der Waals forces (Brown, Jr. and Saxena, 2000). The chains are oriented in parallel and form highly ordered crystalline domains interspersed by more disordered, amorphous regions (Béguin, 1994). The hydroxyl groups at the ends of each cellulose chain have different chemical properties. The number one carbon on the end of a cellulose chain contains an aldehyde hydrate group with reducing activity. On the terminal end of a cellulose chain, the number four carbon is an alcoholic hydroxyl with non-reducing activity.

The packed chains are held together by both intrachain and interchain hydrogen bonds. Their effect over the many residues in the elementary fibril is considerable (Pizzi and Eaton, 1985). The crystalline nature of cellulose implies a structural order in which all of the atoms are fixed in discrete positions with respect to one another. Such feature stiffens individual microfibrils which are sufficiently tight to prevent penetration by even water, let alone enzymes (Lynd et al., 2002). Naturally occurring cellulose fibers are not pure crystalline, and the degree of departure from crystallinity varies. In addition to crystalline and amorphous regions, cellulose fibers contain various types of irregularities such as twists in the microfibrils, or voids such as surface micropores, large pits, and capillaries (Blouin et al., 1970; Cowling, 1975). Degree of crystallization in cellulose varies among species. As crystallinity increases, cellulose becomes increasingly resistant to hydrolysis (Kuhad et al., 1997).
Figure 1.1. Hydrophilic and hydrophobic nature in cellulose chemical structure (Kondo, 2005).
1.2.2. Cellulase

Cellulase is a family of enzymes which hydrolyze cellulose (Emert et al., 1974). Cellulase can be used to hydrolyze cellulosic materials to sugars, which in turn can be fermented to bio-ethanol or used for other bio-based products (Cherry and Fidantsef, 2003). They are commercially used in the textile industry for cotton softening and denim finishing, the detergent market, the food industry for mashing, the pulp and paper industries for deinking, drainage improvement, and fiber modification (Cherry and Fidantsef, 2003; Kirk et al., 2002). Most cellulases are produced by fungi although there are some bacterial sources. The most well known cellulolytic fungi capable of utilizing cellulose as the primary carbon source are *Sclerotium rolfsii*, *Phanerochaete chrysosporium*, *Trichoderma reesei*, and species of *Aspergillus*, *Penicillium*, and *Schizophyllum*. Most commercial cellulase preparations include a β-glucosidase and are produced from either *Trichoderma* and/or *Aspergillus* species (Cherry and Fidantsef, 2003).

1.2.2.1. Classification of Cellulases

Cellulases are classified based on catalytic properties. A typical cellulosic enzyme system contains three major enzymes (Henrissat et al., 1998):

1. **Exo-1,4-β-D-glucanase** (EC 3.2.1.91; cellobiohydrolase), which cleaves cellobiose units from the end of cellulose chains. This class also includes the less common exo-1,4-β-D-glucan hydrolases (EC 3.2.1.74), which liberate D-glucose from the terminal ends of the cellulose chain.

2. **Endo-1,4-β-D-glucanases** (EC 3.2.1.4; EG), which hydrolyze internal β-1,4-glucosidic bonds randomly in the cellulose chain.

3. **1,4-β-D-glucosidases** (EC 3.2.1.21), which hydrolyzes cellobiose to glucose and also cleaves off glucose units from cellooligosaccharides.
1.2.2.2. Cellulase Mechanism

Generally cellulase has been found to have a modular structure that includes both catalytic and carbohydrate-binding modules (CBMs). The CBM binds to the cellulose surface, facilitating cellulose hydrolysis by bringing the catalytic domain to the proximal substrate, insoluble cellulose. CBMs are necessary for the initiation and functioning of exoglucanases. Din et al. (1994) revised the original model of cellulose degradation, which was proposed by Reese et al. (1950), adding a non-catalytic role for CBMs in cellulose hydrolysis. They reported that the “sloughing off” of cellulose fragments from cellulosic surfaces enhances cellulose hydrolysis. Cellulase systems present higher collective activity than the sum of the activities of individual enzymes, a phenomenon known as synergism (Figure 1.2). Synergism is divided to four forms: (1) endo-exo synergy between endoglucanases and exoglucanases, (2) exo-exo synergy between exoglucanases processing from the reducing and non-reducing ends of cellulose chains, (3) synergy between exoglucanases and β-glucosidases that remove cellobiose (and cellodextrins) as end products of the first two enzymes, and (4) intramolecular synergy between catalytic domains and CBMs (Din et al., 1994; Driskill et al., 1999; Lynd et al., 2002). Cellulase systems are not agglomerate enzymes representing the three enzyme groups such as endo-, exoglucanases and β-glucosidases, with/without CBMs, but enzymes that act in a coordinated manner to efficiently hydrolyze cellulose (Tomme et al., 1995). Cellulolytic filamentous fungi such as the aerobic filamentous fungi *Trichoderma reesei* and *Hemicola insolens*, and actinomycete bacteria are capable of penetrating cellulosic substrates with hyphal extensions, presenting their cellulase systems in confined cavities within cellulosic particles (Eriksson, 1990). The production of “free” cellulases, with or without CBMs, may be enough for the efficient hydrolysis of cellulose under these conditions. The best defined cellulase system is that of *T. reesei*, initially called *T. viride* (Lynd et al., 2002). The cellulase system from *T. reesei* includes at least two exoglucanases (CBHI and CBHII), five endoglucanases (EGI, EGII,
Figure 1.2. Schematic representation of sequential stages of cellulase (Lynd et al., 2002).
EGIII, EGIV, and EGV), and two β-glucosidases (BGLI and BGLII).

1.2.2.3. Enzymatic Hydrolysis of Lignocellulosic Material

The hydrolysis of lignocellulosic material by cellulase is an expensive step accounting for approximately 30% of the total cost of bio-refinery due to the enzyme cost. Cellulase loading should be minimized but this increases the time required to complete the hydrolysis. The performance of cellulase in a bio-processing step on lignocellulosic material depends on a number of factors such as the presence and structure of lignin, temperature, pH, and substrate concentration. The use of slurry from a pretreated lignocellulosic material causes more problems than a pure cellulose. There is a lower substrate concentration because of the presence of other components (Gregg and Saddler, 1995). The enzyme performance is significantly reduced by end product inhibition and by sugars, and other degradation products produced during the pretreatment. To overcome some of these problems a washing step may be employed (Tenborg et al., 2001a; Tenborg et al., 2001b). The continuous removal of sugars has been tested using ultrafiltration (Ishihara et al., 1991; Gan et al., 2002). Similarly, a strategy to recycle the cellulases using ultrafiltration after hydrolysis has been investigated in order to reduce the cost of lignocellulose hydrolysis (Singh et al., 1991; Ramos et al., 1995).

1.2.2.4. Cellulase Inhibition

Any process utilizing cellulase enzymes must deal with end-product inhibition. Quantitative research has been performed to elucidate the mechanisms of end-product inhibition in the cellulase systems (Philippidis et al., 1993; Xiao et al., 2004). However, the types of inhibition presented by cellulases are not straightforward (Holtzapple, 1990). Although most studies have shown that end products of cellulase systems generally cause competitive inhibition, some cellulases are noncompetitively or uncompetitively inhibited. This may be because the cellulase system includes multi-enzyme components, moreover, they act synergistically.
Xiao et al. (2004) investigated the inhibitory effects of sugars; glucose, mannose, galactose, and xylose on both cellulase and β-glucosidase. Glucose supplementation of 20 g/L produced 53% inhibition of β-glucosidase at a cellobiose concentration of 20 g/L. However, cellulase activity produced a higher degree of inhibition than glucose supplementation on β-glucosidase. Simple sugars such as galactose, mannose, and xylose did not inhibit β-glucosidase at concentrations up to 100 g/L, whereas, these sugars have significant inhibitory effects on cellulase activity (Xiao et al., 2004). Previously researchers had reported that the addition of these sugars at more than 10 g/L was inhibitory to β-glucosidase (Saha et al., 1994; Yun et al., 2001).

Bezerra and Dias (2005) described an inhibitory effect of ethanol and cellobiose on both cellulase and purified exoglucanase Cel7A from *T. reesei*. Ethanol inhibited a crude cellulase in a noncompetitive manner at a lower concentration than on exoglucanase Cel7A, where it was a competitive inhibitor. Adsorption of cellulases to cellulose, especially cellobiohydrolases, is interfered with ethanol. Ethanol also modifies the cooperative effect between cellobiohydrolases and endoglucanases (Caldini et al., 1994). Cellobiose is a strong competitive inhibitor of exoglucanase Cel7A and a noncompetitive inhibitor of crude cellulase. Cellobiose inhibition is stronger than ethanol inhibition both for crude cellulases and exoglucanase Cel7A.

Holtzapple et al. (1990) reported that glucose, cellobiose and the solvents-ethanol, acetone and butanol were noncompetitive inhibitors and δ-gluconolactone was a mixed inhibitor of cellulase from *T. reesei*.

### 1.2.3. Cellobiose

Cellobiose is a glucan disaccharide linked β-1,4 and is a repeating unit of cellulose. It has enormous biotechnological potential as a renewable product for use in food, cosmetic, and pharmaceutical industries. Enzymes, capable of breaking down cellobiose, are absent in the human small
intestine. Nakamura (2004) confirmed that cellobiose may be slowly hydrolyzed by intestinal lactase in an in vitro study with rat small intestinal brush border membrane vesicles. He also estimated cellobiose bioavailability using cellobiose tolerance tests and breath hydrogen excretion tests from 10 healthy young women and concluded that cellobiose was poorly hydrolyzed or fermented by intestinal microbes, estimating a caloric value of 2 kcal/g in humans. In the prebiotic study of Sanz et al. (2005), cellobiose resulted in a higher PI (prebiotic index), indicating a relationship between changes in the “beneficial” and “undesirable” elements within the microflora, than a currently used prebiotic agent, fructooligosaccharides. Correspondently, Yamazaki and Ichiro (2007) demonstrated cellooligosaccharides containing over 70% cellobiose are metabolized by Bifidobacterium and lactic acid bacteria and are not utilized by *Clostridium perfringens* and could be used for pharmaceuticals and functional foods. Moreover, cellobiose fermentation in human fecal slurry gave a much higher content of butyric acid which has been shown to have cancer-preventing properties (Calabresse et al., 1993; Riggs et al., 1977) than fructooligosaccharides (Sanz et al., 2005). Orally administered cellobiose reduced neural fat in the liver and total cholesterol, proposing use as potential drugs for prevention and treatment of life style-related disease along with cellooligosaccharides (Yamazaki and Ibuki, 2008). Cellobiose was also utilized for the production of lactic acid by *Lactobacillus delbrueckii* mutant (Adsul et al., 2007).

There are some reports on new types of oligomers containing cellobiose as a component produced by transglycosylation reactions (Lee et al., 2001; Morales et al., 2001). Acarbose analogues containing cellobiose were prepared by the reaction of acarbose and maltose catalyzed by a *Bacillus stearothermophilus* maltogenic amylase (Lee et al., 2001). Cellobiose-acarbose analogues showed potential as inhibitors for β-glucosidase, whereas acarbose did not. Morales *et al.* (2001) produced the oligosaccharides α-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-D-glucopyranose, α-D-
glucopyranosyl-(1→6)-[β-D-glucopyranosyl-(1→4)]-D-glucopyranose, α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→6)-[β-D-glucopyranosyl-(1→4)]-D-glucopyranose, and α-D-glucopyranosyl-(1→3)-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→6)-[β-D-glucopyranosyl-(1→4)]-D-glucopyranose using cellobiose as an acceptor in the reaction catalyzed by alternansucrase (EC 2.4.1.140) from *Leuconostoc mesenteroides* NRRL B-23192.

In this study, we quantified the degree and determined the pattern of inhibition of the cellulase system by gluconolactone, gluconic acid, or glucose oxidase, and analyzed productivity of cellobiose using Box-Behnken design.

### 1.3. MATERIALS AND METHODS

#### 1.3.1. Substrates, Enzymes, and Chemicals

Avicel™ Type PH 101 (FMC, Philadelphia, PA) was swollen with phosphoric acid by the method of Rignall *et al.* (2002) prior to evaluation for the production of cellobiose. Ammonia treated sugarcane bagasse was kindly obtained from Dr. G. DeQueiroz (Audubon Sugar Institute, LSU Ag Center). The treated bagasse contained 58.2% glucan calculated from cellulase digestibility based on glucose and cellobiose concentration obtained after 48 hour hydrolysis. This glucan concentration was taken to reflect the cellulose content of the ammonia treated bagasse. The cellulosic substrates-phosphoric acid swollen Avicel™ and ammonia treated sugarcane bagasse were moistened ten times with 200 mM sodium citrate buffer, pH 5.2 prior to hydrolysis. Cellobiose (Sigma-Aldrich, St. Louis, MO) was employed as the substrate for the β-glucosidase assay. Cellulase, containing the complete cellulase system of exo- and endo-glucanase and β-glucosidase, synthesized by a *Trichoderma viride* strain, and glucose oxidase were both purchased from Sigma-Aldrich (St. Louis, MO). The chemicals used as inhibitors, gluconolactone and gluconic acid were also from the same source (Sigma-Aldrich, St. Louis, MO).
1.3.2. Enzyme Activities

Cellulase activity was determined as FPU (filter paper unit) according to a NREL procedure (NREL accessed June 6, 2005). The unit definition of glucose oxidase followed that of the manufacturer where a unit is the amount that oxidizes 1.0 µmole of β-D-glucose to D-gluconolactone and H₂O₂ in one min at pH 5.1 and 37°C. The β-glucosidase activity was determined using cellobiose as a substrate.

1.3.3. Enzyme Hydrolysis

For inhibition studies, one percent of phosphoric acid swollen Avicel™ was used as a substrate and 0.46 FPU/ml (45.8 FPU/g glucans) of cellulase was used with varying concentrations of inhibitors, 0.1-10% gluconolactone, 0.1-5% gluconic acid, or 0.51-510 U/ml of glucose oxidase. The activity was measured after 30 minutes reaction to minimize end product interference of cellulase and β-glucosidase. Several experimental groups were tested to optimize cellobiose production. They are defined and their conditions described below in the section of a Box-Behnken experimental design. All studies were performed at 37°C and 150 rpm in 200 mM sodium citrate buffer, pH 5.2 containing 6 µg/mL of tetracycline and 3 µg/mL of cyclohexamide.

1.3.4. Quantification of Sugars

The concentrations of cellobiose and glucose were measured using high-performance liquid chromatograph (HPLC) equipped with a refractive index detector. An Aminex-HPX-87K column (Bio-Rad Lab., Hercules, CA) was used with 0.01 M K₂HPO₄ as mobile phase. It was run at a constant rate of 0.6 ml/min at 85°C.

1.3.5. Box-Behnken Experimental Design

A four-factor and three-level Box-Behnken design was applied for optimization of cellobiose production using Design Expert 7.13 software (Stat-Ease, Inc.). Several factors, the amount of cellulose, cellulase, each inhibitor and reaction time, were used to prepare each of the 29 formulations given in
Tables 1.2 and 1.3. The high, medium, and low levels were selected based on preliminary experimentation. Optimization was performed using a desirability function to obtain the levels of $X_1$, $X_2$, $X_3$, and $X_4$.

The behavior of the system has been explained by the following quadratic model equation (Box and Behnken, 1960);

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4$$

where $Y$ is predicted response, $\beta_0$ intercept, $\beta_1$, $\beta_2$, $\beta_3$, and $\beta_4$ linear coefficient, $\beta_{11}$, $\beta_{22}$, $\beta_{33}$, and $\beta_{44}$ squared coefficients and $\beta_{12}$, $\beta_{13}$, $\beta_{14}$, $\beta_{23}$, $\beta_{24}$, and $\beta_{34}$ interaction coefficients. A total of 29 experiments were necessary to determine the 15 coefficients of model.

### 1.4. RESULTS AND DISCUSSION

#### 1.4.1. Inhibition Study on a Cellulase System

The inhibition patterns produced by glucose oxidase on cellulase and $\beta$-glucosidase activity were compared (Figure 1.3; Day et al., 2008). The inhibition pattern was similar for both cellulase and $\beta$-glucosidase. The addition of greater than 0.1275 U/ml glucose oxidase reduced both cellulase and $\beta$-glucosidase activity. In the presence of 51 U/ml glucose oxidase, activity was 11% of the original for $\beta$-glucosidase and 26% of the original for cellulase showing the saturation for the inhibitor, glucose oxidase. An oxidized product of glucose by glucose oxidase, gluconic acid (Duke et al., 1969; Singh and Kumar, 2007) showed similar inhibition patterns for both cellulase and $\beta$-glucosidase (Figure 1.4). At a concentration of 1.0% gluconic acid, a 78% decrease in cellulase activity and 90% decrease in $\beta$-glucosidase activity were observed. Gluconolactone also produced equivalent degrees of inhibition on both cellulase and $\beta$-glucosidase activity (Figure 1.5). Both enzymes lost approximately 70% of their activity in the presence of 0.02% gluconolactone. Gluconolactone has been reported to be a selective $\beta$-
Table 1.2. Independent variables in a Box-Behnken design.

<table>
<thead>
<tr>
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<th>Variable</th>
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<td>$X_1$:</td>
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<td>$X_2$:</td>
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<td>$X_3$:</td>
<td>Glucose oxidase (U/ml)</td>
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<td>Gluconic acid (%)</td>
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<td>Gluconolactone (%)</td>
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<td>$X_4$:</td>
<td>Reaction time (h)</td>
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<tr>
<td>Run</td>
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Figure 1.3. Changes of relative activities of cellulase and β-glucosidase in the presence of glucose oxidase. Relative activity is the % of enzyme activity in the presence of the test concentration of glucose oxidase to the enzyme activity of the reaction in the absence of glucose oxidase. For the determination of β-glucosidase activity, 0.3% cellobiose was reacted with 0.46 FPU/ml cellulase in 200 mM sodium citrate buffer (pH 5.2) at 37°C, for 30 min. For the determination of cellulase activity, 1% phosphoric acid swollen Avicel™ was reacted with 0.46 FPU/ml cellulase in the presence of 0, 0.002, 0.008, 0.0319, 0.1275, 0.51, 5.1, and 51 U/ml glucose oxidase in 200 mM sodium citrate buffer (pH 5.2) at 37°C for 30 min.
Figure 1.4. Relative activity of cellulase and β-glucosidase in the presence of gluconic acid. Relative activity is the % of enzyme activity at test concentration of gluconic acid to the enzyme activity in the absence of gluconic acid. Inserted figure is the relative activity in the presence of gluconic acid between 0 and 0.10%. For the determination of β-glucosidase activity, 0.3% cellobiose was reacted with 0.46 FPU/ml cellulase in 200 mM sodium citrate buffer (pH 5.2) at 37°C for 30 min. For the determination of cellulase activity, 1% phosphoric acid swollen Avicel™ was reacted with 0.46 FPU/ml cellulase in the addition of 0, 0.02, 0.04, 0.10, 1.0, and 5.0% gluconic acid in 200 mM sodium citrate buffer (pH 5.2) at 37°C for 30 min. Error bars show the standard deviation error of the mean.
Figure 1.5. Relative activities of cellulase and β-glucosidase in the presence of gluconolactone. Relative activity is the % of enzyme activity at test concentration of gluconolactone to the enzyme activity at 0% gluconolactone addition. For the determination of β-glucosidase activity, 0.3% cellobiose was reacted with 0.46 FPU/ml cellulase in 200 mM sodium citrate buffer (pH 5.2) at 37°C for 30 min. For the determination of cellulase activity, 1% phosphoric acid swollen Avicel™ was reacted with 0.46 FPU/ml cellulase in the addition of 0, 0.02, 0.04, and 0.06% gluconolactone in 200 mM sodium citrate buffer (pH 5.2) at 37°C for 30 min. Error bars show the standard deviation error of the mean.
glucosidase inhibitor among a cellulase system, although its cost negates industrial use (Holtzapple et al. 1990; Philippidis et al. 1993). Philippidis et al. (1993) reported that the presence of 3 g/L gluconolactone produced a 70% loss of β-glucosidase activity but left intact cellulase activity at 10 g/L of cellobiose and 60 g/L of cellulose. Less than 4% of β-glucosidase activity and 87% of cellulase activity remained in the presence of 7.5 g/L gluconolactone with 20 g/L of cellulose (Holtzapple et al., 1990). As a result, our data suggested that an inhibitor such as glucose oxidase, gluconolactone, or gluconic acid selectively inhibit β-glucosidase in a cellulase system. It has potential that exoglucanase and endoglucanase in cellulase synergistically act on cellulose hydrolysis when uncoupled from the hydrolytic action of β-glucosidase to produce increased amounts of cellobiose in a relatively short time.

Gluconic acid and gluconolactone act as mixed inhibitors since the lines intersect in the lower left quadrant (Figure 1.6 and Figure 1.7). The Ki values were 2.35 mg/ml at 5% gluconic acid concentration and 3.5 mg/ml at 1% gluconolactone concentration. Our inhibition studies agree with others in that gluconolactone and gluconic acid exhibit mixed inhibition (Dixon et al., 1979; Holtzapple et al., 1990). Mixed-type inhibition is sometimes called noncompetitive inhibition. Mixed inhibition type is represented in the following equilibria;

The mixed type inhibition interprets that the presence of inhibitor (I) on the enzyme changes the dissociation constant for substrate (S) from $K_s$ to $\alpha K_s$ (Segel, 1976).

However, kinetic studies of sugars and their analogues on cellulase enzymes have been debated because the inhibition mechanism is largely dependent on the source and concentration of enzymes, the
Figure 1.6. Lineweaver-Burk plots for gluconic acid. The reaction was performed with 1% phosphoric acid swollen Avicel\textsuperscript{TM}, 0.46 FPU/ml cellulase, and 5% gluconic acid in 200 mM sodium citrate buffer (pH 5.2) at 37°C for 30 min. As control, the reaction condition was same above except for glucononic acid absence. The inhibition degree was calculated based on the released glucose amount from 1% phosphoric acid Avicel\textsuperscript{TM}. 
Figure 1.7. Lineweaver-Burk plots for gluconolactone. The reaction was performed with 1% phosphoric acid swollen Avicel™, 0.46 FPU/ml cellulase, and 1% gluconolactone in 200 mM sodium citrate buffer (pH 5.2) at 37°C for 30 min. As control, the reaction condition was same above except for gluconolactone absence. The inhibition degree was calculated based on the released glucose amount from 1% phosphoric acid Avicel™.
conditions of hydrolysis, and even the method of analysis of products (Gusakov and Sinitsyn, 1992; Dekker, 1988; Montero and Romeu, 1992).

1.4.2. Effects of Inhibitors on Hydrolysis of Cellulose

Cellobiose concentration was monitored over the course of 38 hours during the hydrolysis of cellulose in the presence of glucose oxidase, gluconic acid, or gluconolactone at 37°C. A considerable increase in cellobiose in solution was observed with more than 0.51 U/ml loading of glucose oxidase (Figure 1.8). The concentration of cellobiose in solution increased with increasing glucose oxidase loading up to 12 hours. Saturation of the reaction with glucose oxidase seem to be somewhere around 51 U/ml glucose oxidase. The highest concentration of cellobiose achieved was 1.93 mg/ml in 24 hour hydrolysis in the presence of 51 U/ml of glucose oxidase and it was eight times higher than when glucose oxidase was absent. This increase may result from the combined effect of decreased cellulase inhibition by the removal of glucose by oxidization or from β-glucosidase inhibition by gluconic acid, a product of glucose oxidase. Without glucose oxidase, the concentration of cellobiose was only 0.24 mg/ml. A decrease in cellobiose in solution was observed after it reached its highest point in samples with glucose oxidase. This is why an increase in cellobiose in solution might inhibit exoglucanase and endoglucanase as a product inhibition (Xiao et al., 2004; Bezerra and Dias, 2005; Holtzapple et al., 1990), resulting in interference of cellobiose release.

The log increases in glucose oxidase concentrations in the cellulase hydrolysis mixture produced continuous increases in cellobiose concentrations in solution at 12 hour hydrolysis (Figure 1.9; Day et al., 2008). A large excess of glucose oxidase suppressed the production of cellobiose. High concentrations of glucose oxidase may interfere with the cellulase hydrolysis reactions obstructing the approach of the cellulase to cellulose. Glucose yields decreased rapidly with the addition of increased glucose oxidase showing that glucose oxidase effectively oxidized glucose released.
Figure 1.8. Impact of various glucose oxidase loadings on the production of cellobiose during 1% Avicel™ hydrolysis. The percentage of cellobiose yield was calculated at released cellobiose concentration to 1% phosphoric acid swollen Avicel™. The reaction was performed with 1% phosphoric acid swollen Avicel™ and 0.46 FPU/ml cellulase in the presence of 0, 0.51, 5.1, and 51 U/ml glucose oxidase in 200 mM sodium citrate buffer (pH 5.2) at 37°C. The cellobiose concentration was tested at 0, 12, 24, and 38 h.
Figure 1.9. Impact of various glucose oxidase loadings on the production of cellobiose and glucose during 1% Avicel™ hydrolysis at 12 hour hydrolysis. The percentage of cellobiose yield was calculated at released cellobiose concentration to 1% phosphoric acid swollen Avicel™. The reaction was performed with 1% phosphoric acid swollen Avicel™ and 0.46 FPU/ml cellulase in the presence of 0.51, 5.1, 51, and 510 U glucose oxidase in 200 mM sodium citrate buffer (pH 5.2) at 37°C.
Gluconic acid addition to the reaction mixtures produced an increase in cellobiose during cellulose hydrolysis (Figure 1.10). The addition of 5% or 10% gluconic acid gave a similar pattern of cellobiose yield increases. The highest yield of cellobiose was 2.97 mg/ml in the presence of 5% gluconic acid over a 38 hour hydrolysis. This was ten times higher than the control reaction mixture (without gluconic acid).

There were large differences in the amount of cellobiose produced when gluconolactone was added to reaction mixtures containing 1% Avicel™ and 0.46 FPU/ml cellulase (Figure 1.11). The highest yield of cellobiose (3.06 mg/ml) was achieved after 24 hours of cellulose hydrolysis by supplementing with 1% gluconolactone. Excess gluconolactone (more than 1%) inhibited both cellobiose and glucose production. The addition of 0.1% gluconolactone was insufficient for consistent production of cellobiose, since cellobiose concentration decreased rapidly with time.

1.4.3. Box-Behnken Experimental Design

Optimization of cellobiose production from cellulose by cellulase in the presence of each inhibitor was achieved by employing a Box-Behnken design. The response surface methodology (RSM) was used to study the effect of interaction among independent variables on the production of cellobiose. RSM has been used as an effective statistical technique in the optimization of several biotechnological processes. RSM evaluates the effects of more than two independent variables on the dependent factor using multiple regression and correlation analysis. It has some advantages—the reduced number of experimental runs required to generate sufficient information to accept a statistical result and faster research method than classical tests (Kim and Day, 2007; Gunawan et al., 2005; Jeong and Park, 2007).

In order to estimate the cellobiose yield from cellulose by cellulase modification system, the estimative response model equations were as follow:

\[ Y = -77.01 + 45.32X_1 - 0.07X_2 + 4.64X_3 + 3.19X_4 + 1.18X_1X_2 - 0.18X_1X_3 - 0.22X_1X_4 - 0.05X_2X_3 - \]
Figure 1.10. Impact of gluconic acid concentration on the production of cellobiose during 1% Avicel™ hydrolysis. The percentage of cellobiose yield was calculated at released cellobiose concentration to 1% phosphoric acid swollen Avicel™. The reaction was performed with 1% phosphoric acid swollen Avicel™ and 0.46 FPU/ml cellulase in the presence of 0, 1, 5, and 10% gluconic acid in 200 mM sodium citrate buffer (pH 5.2) at 37°C. The cellobiose concentration was tested at 0, 12, 24, and 38 h.
Figure 1.11. Impact of gluconolactone concentration on the production of cellobiose during 1% Avicel™ hydrolysis. The percentage of cellobiose yield was calculated at released cellobiose concentration to 1% phosphoric acid swollen Avicel™. The reaction was performed with 1% phosphoric acid swollen Avicel™ and 0.46 FPU/ml cellulase in the presence of 0, 0.1, 1.0, 5.0, and 10.0% gluconolactone in 200 mM sodium citrate buffer (pH 5.2) at 37°C. The cellobiose concentration was tested at 0, 12, 24, and 38 h.
0.0007X_2X_4 - 0.02X_3X_4 - 4.67X_1^2 - 0.22X_2^2 - 0.05X_3^2 - 0.05X_4^2 for a glucose oxidase model;

\[ Y = -10.43 + 2.63X_1 + 16.76X_2 + 8.16X_3 + 2.41X_4 + 0.86X_1X_2 - 0.77X_1X_3 + 0.38X_1X_4 + 0.75X_2X_3 - 0.06X_2X_4 - 0.12X_3X_4 - 1.56X_1^2 - 1.95X_2^2 - 0.38X_3^2 - 0.06X_4^2 \]

for a gluconic acid model;

\[ Y = -25.12 + 20.43X_1 + 4.05X_2 + 11.03X_3 + 4.48X_4 + 0.59X_1X_2 + 0.25X_1X_3 + 0.05X_1X_4 + 0.97X_2X_3 + 0.04X_2X_4 - 0.07X_3X_4 - 3.66X_1^2 - 0.76X_2^2 - 2.22X_3^2 - 0.10X_4^2 \]

for a gluconolactone model

where \( Y \) is the response factor, relative cellobiose yield (%), \( X_1, X_2, X_3, \) and \( X_4 \) are the real values of the independent factors; cellulose concentration (%), cellulase concentration (FPU), inhibitor (U/ml for glucose oxidase and % for others), and reaction time (h). Table 1.4 presents the actual and predicted values of cellobiose production on the basis of the experimental design, and their values agreed reasonably. The results were analyzed by multiple regression and the coefficients were tested for their significance through regression analysis. The coefficients were evaluated through regression analysis. For the glucose oxidase model, two linear coefficients of \( X_1 \) and \( X_3 \), and one quadratic coefficient, \( X_1^2 \) were determined to be significant at \( p<0.05 \) (Table 1.5). The glucose oxidase model is highly significant as an evidence from \( F_{\text{model}} = 10.44 \) and a low probability value (<0.0001) in the analysis of variance of this model. A gluconic acid model showed that three linear coefficient of \( X_1, X_2, X_3 \), one cross-product coefficient of \( X_2X_3 \) and two quadratic coefficients of \( X_2^2 \) and \( X_4^2 \) were determined to be significant (Table 1.6). The gluconic acid model is also highly significant as an evidence from \( F_{\text{model}} = 7.38 \) and a low probability value (0.0003) in the analysis of variance of this model (Table 1.6). Also, the F value of 0.86 for the lack of fit demonstrates that it is not significant relative to the pure error according to our results of analysis of factors, because non-significant lack of fit is a good indication that the model fits the actual relationships of the reaction parameters within the selected ranges. Concerning the \( p \)-value of the coefficients for the gluconolactone model, \( X_1, X_2, X_3, X_2X_3, X_1^2, X_2^2, X_3^2 \), and \( X_4^2 \) have significant effects at \( p<0.05 \) for the prediction of the cellobiose pro-
Table 1.4. Relative observed and predicted cellobiose yield (%) in the presence of inhibitors for 3-level-4-factor response surface analysis.

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<th>$X_2$: Gluconic Acid</th>
<th>$X_3$: Gluconolactone</th>
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<td>Observed $Y$</td>
<td>Observed $Y$</td>
<td>Predicted $Y$</td>
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*aInformation is mentioned in Table 1.3.*
Table 1.5. ANOVA for response surface for cellobiose production in the presence of glucose oxidase.

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<sup>a</sup>Probability>F, level of significance.  
<sup>b</sup>Values of “probability > F” less than 0.05 indicate model terms are significant. Values greater than 0.1 indicate the model terms are not significant.
Table 1.6. ANOVA for response surface for cellobiose production in the presence of gluconic acid.

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<sup>a</sup>Probability > F, level of significance.
<sup>b</sup>Values of “probability > F” less than 0.05 indicate model terms are significant. Values greater than 0.1 indicate the model terms are not significant.
duction (Table 1.7). The model coefficients and probability values showed that the gluconolactone model is highly significant, as evident from the $F$ ($F_{model} = 4.40$) and a very low probability value (0.0045).

Figure 1.12 shows the response of surface plots representing the effect of four factors—the concentration of cellulose, cellulase, and glucose oxidase, reaction time and their reciprocal interactions with cellobiose yield by holding two of the factors constant at a middle level. Figure 1.12a shows the effects of different concentrations of cellulase and cellulose on cellobiose production with a constant reaction time (24 hours) and glucose oxidase concentration (25.75 U/ml). The maximal cellobiose production was attained using 5% cellulose and 10 FPU cellulase. The effects of different concentrations of glucose oxidase and cellulose on cellobiose production with 5.1 FPU cellulase and a 24 hour reaction time are shown in Figure 1.12b. Maximal cellobiose production was seen at a cellulose concentration between 4 and 5% and glucose oxidase loading between 25.76 and 38.38 U/ml. Figure 1.12c represents the relationship between different concentrations of cellulose and reaction times on the cellobiose production, at a fixed concentration of 5.1 FPU cellulase and 25.75 U glucose oxidase. Across the entire range of reaction times, maximal cellobiose production was obtained at concentrations of cellulose of between 4 and 5%. As shown in Figure 1.12d, cellobiose production was affected only slightly by cellulase concentrations, the highest cellobiose production was achieved using glucose oxidase loadings ranged from 25.75 to 38.36 U/ml with 3% cellulose and a 24 hour reaction time. The effects of cellulase concentrations and reaction times on cellobiose production are shown in Figure 1.12e. The cellobiose production was sensitive to cellulase concentrations and reaction times. The optimal yield was obtained at 5.1 FPU cellulase and a 24 hour reaction time. Figure 1.12f shows the effects of different glucose oxidase concentrations and reaction times on cellobiose production with the fixed concentrations of cellulose (3%) and cellulase (5.10 FPU). An increase in the amount of introduced glucose oxidase
Table 1.7. ANOVA for response surface for cellobiose production in the presence of gluconolactone.

<table>
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<sup>a</sup>Probability > F, level of significance.
<sup>b</sup>Values of “probability > F” less than 0.05 indicate model terms are significant. Values greater than 0.1 indicate the model terms are not significant.
Figure 1.12. Response surface plots illustrating the effect of varying concentrations of Avicel™, cellulase, and glucose oxidase, and reaction time on their reciprocal interactions with cellobiose production. Other factors are held constant at 25.75 U glucose oxidase and 24 h reaction time (a); 5.10 FPU cellulase and 24 h reaction time (b); 5.10 FPU cellulase and 25.75 U glucose oxidase (c); 3% Avicel™ and 24 h reaction time (d); 3% Avicel™ and 25.75 U glucose oxidase (e); 3% Avicel™ and 5.10 FPU cellulase (f). All reactions were performed in 200 mM sodium citrate buffer (pH 5.2) at 37°C and 150 rpm.
resulted in a linear increase in cellobiose up to 38.38 U/ml glucose oxidase. As a result, optimal value of the selected factors were determined as follow: $X_1$ (cellulose) = 4%, $X_2$ (cellulase) = 8.2 FPU, $X_3$ (glucose oxidase) = 17.3 U/ml, and $X_4$ (reaction time) = 16 hours.

Figure 13 elucidates the relationship between the independent and dependent variables in the gluconic acid model. The best results were shown in cellulase loading between 2.65 and 7.55 FPU, whereas the increase in cellulose concentration did not increase cellobiose yield at 5.25% gluconic acid and 24 hour reaction time (Figure 1.13a). The effects of different gluconic acid concentrations and cellulose on the production of cellobiose at 5.1 FPU cellulase and 24 hour reaction time are shown in Figure 1.13b. Both increases in the amount of gluconic acid and cellulose resulted in a linear increase in cellobiose yield. Maximal production was conducted using a high gluconic acid and cellulose concentration. Figure 1.13c represents the effects of different concentration of cellulose and reaction time on cellobiose production at 5.1 FPU cellulase and 5.25% gluconic acid. Increase in reaction time resulted in rapid decrease in cellobiose production whereas different concentrations of cellulose introduced did not largely change cellobiose production. Cellobiose production was affected more by the amount of cellulase loading than reaction time at 3% Avicel™ and 5.25% gluconic acid (Figure 1.13d). Within the entire range of cellulase amount and reaction time, the maximal cellobiose production was accomplished at cellulase amount between 5.10 and 7.55 FPU. As shown in Figure 1.13e, the high concentration of gluconic acid (7.65-10%) resulted in an increased cellobiose production with a lower reaction time (18 hours) at 3% cellulose and 5.10 FPU cellulase. Figure 1.13f shows the effects of different concentrations of gluconic acid and cellulase on cellobiose production with a constant reaction time (24 hours) and cellulose concentration (3%).

The optimal yield was obtained at 5.1 FPU cellulase and high concentration of gluconic acid (10%). Therefore, the optimal conditions for the production of cellobiose were obtained as follow:
Figure 13. Response surface plots illustrating the effect of varying concentrations of Avicel™, cellulase, and gluconic acid, and reaction time on their reciprocal interactions with cellobiose production. Other factors are held constant at 5.25% gluconic acid and 24 h reaction time (a); 5.10 FPU cellulase and 24 h reaction time (b); 5.10 FPU cellulase and 5.25% gluconic acid (c); 3% Avicel™ and 5.25% gluconic acid (d); 3% Avicel™ and 5.10 FPU cellulase (e); 3% Avicel™ and 24 h reaction time (f). All reactions were performed in 200 mM sodium citrate buffer (pH 5.2) at 37°C and 150 rpm.
$X_1$ (cellulose) = 4%, $X_2$ (cellulase) = 2.5 FPU, $X_3$ (gluconic acid) = 9.5%, and $X_4$ (reaction time) = 22 hours.

Figure 1.14 shows the relationship in the presence of gluconolactone between the independent and dependent variables elucidated using contour and response surface plots. The best results are restricted to Avicel\textsuperscript{TM} concentration between 2 and 4%, cellulase loading between 2.55 and 7.55 FPU, gluconolactone concentration between 2.55 and 3.77, and reaction time between 18 and 30 hours. They present the narrowest ranges of values that generated the zone of high values of the desirability function. Finally, the optimal conditions for the production of cellobiose were determined as follow: $X_1$ (cellulose) = 4%, $X_2$ (cellulase) = 7.6 FPU, $X_3$ (gluconolactone) = 3.9%, and $X_4$ (reaction time) = 25 hours.

1.4.4. Production of Cellobiose from Lignocellulosic Biomass

Cellobiose production was tested using the optimal conditions obtained from Box-Behnken design with phosphoric acid swollen Avicel\textsuperscript{TM} and ammonia treated sugarcane bagasse as lignocellulosic biomass. The concentration of ammonia treated sugarcane bagasse was introduced with the same cellulose content into the reaction mixture. Cellobiose production of 9.48 mg/ml from phosphoric acid swollen Avicel\textsuperscript{TM} and 9.84 mg/ml from ammonia treated sugarcane bagasse was observed in glucose oxidase mixtures whereas non-glucose oxidase reaction mixtures produced 1.2-1.3 mg/ml of cellobiose (Figure 1.15). Also, enzymatic conditions in the presence of gluconic acid showed increased cellobiose production of 8.76 mg/ml from phosphoric acid swollen Avicel\textsuperscript{TM} and 9.24 mg/ml from ammonia treated sugarcane bagasse whereas non-gluconic acid added reaction mixtures produced 1.2-1.3 mg/ml of cellobiose (Figure 1.16). The enzymatic modification system with gluconolactone showed increased cellobiose production of 12.5 mg/ml from phosphoric acid swollen Avicel\textsuperscript{TM} and 11.03 mg/ml from ammonia treated sugarcane bagasse whereas non-gluconolactone added reaction mixtures produced 1.2-1.3 mg/ml of cellobiose (Figure 1.17). In our study, the maximum yield (31.25%) of cellobiose based on
Figure 1.14. Response surface plots illustrating the effect of varying concentrations of Avicel™, cellulase, and gluconolactone, and reaction time on their reciprocal interactions with cellobiose production. Other factors are held constant at 2.55% gluconolactone and 24 h reaction time (a); 5.10 FPU cellulase and 24 h reaction time (b); 3% Avicel™ and 24 h reaction time (c); 3% Avicel™ and 2.55% gluconolactone (d); 3% Avicel™ and 5.1 FPU cellulase (e); 5.1 FPU cellulase and 2.55% gluconolactone (f). All reactions were performed in 200 mM sodium citrate buffer (pH 5.2) at 37°C and 150 rpm.
Figure 1.15. Cellobiose production from cellulose using cellulase modification system with glucose oxidase. The production of cellobiose from 4% phosphoric acid swollen Avicel™ was determined with reaction mixtures containing 8.2 FPU cellulase and 17.3 U glucose oxidase after 16 h hydrolysis. The production of cellobiose from 7% ammonia treated bagasse was evaluated with reaction mixtures containing 8.2 FPU cellulase and 17.3 U glucose oxidase after 16 h hydrolysis. The control reaction mixtures contained the same formulations except for glucose oxidase absence. All reaction mixture was performed in 200 mM sodium citrate buffer (pH 5.2) at 37°C and 150 rpm. Error bars show the standard deviation error of the mean.
Figure 1.16. Cellobiose production from cellulose using cellulase modification system with gluconic acid. The production of cellobiose from 4% phosphoric acid swollen Avicel™ was determined with reaction mixtures containing 2.5 FPU cellulase and 9.5% gluconic acid after 22 h hydrolysis. The production of cellobiose from 7% ammonia treated bagasse was evaluated with reaction mixtures containing 2.5 FPU cellulase and 9.5% gluconic acid after 25 h hydrolysis. The control reaction mixtures contained the same formulations except for gluconic acid absence. All reaction mixture was performed in 200 mM sodium citrate buffer (pH 5.2) at 37°C and 150 rpm. Error bars show the standard deviation error of the mean.
Figure 1.17. Cellobiose production from cellulose using cellulase modification system with gluconolactone. The production of cellobiose from 4% phosphoric acid swollen Avicel™ was determined with reaction mixtures containing 7.6 FPU cellulase and 3.9% gluconolactone after 25 h hydrolysis. The production of cellobiose from 7% ammonia treated bagasse was evaluated with reaction mixtures containing 7.6 FPU cellulase and 3.9% gluconolactone after 25 h hydrolysis. The control reaction mixtures contained the same formulations except for gluconolactone absence. All reaction mixture was performed in 200 mM sodium citrate buffer (pH 5.2) at 37°C and 150 rpm. Error bars show the standard deviation error of the mean.
its initial cellulose substrate was achieved with 4% substrate, 7.6 FPU cellulase, and 3.9% gluconolactone. Increasing cellobiose concentrations inhibited cellulase activity, blocking further increases in cellobiose production. Cellobiose has been known as a strong inhibitor of cellulase. Especially, Cel 7A which is one of the exoglucanases and the most abundant cellulase produced by *T. reesei* is inhibited by cellobiose (Gruno et al., 2004). Other researchers also agreed that cellobiose exhibits a great impact on cellulase activity (Gusakov and Sinitsyn, 1992; Holzapple et al., 1990; Xiao et al., 2004; Dekker, 1986). Holtzapple et al. (1990) hypothesized that the inhibitor binds to the active site as is seen in the relative values of the cellobiose and glucose binding constants regardless of the inhibition pattern. Also, they reported that the cellobiose binding constant was 3-14 times greater than the glucose binding constants because the cellulase active site binds β-1,4-linked glucose polymers, longer glucose polymers have more sites of attachment and hence a greater binding constant.

Lignocellulose substrate-ammonia treated sugarcane bagasse produced less cellobiose than phosphoric acid swollen Avicel™ on the basis of yield (%) although it was superior to the control. The addition of glucose oxidase yielded 23.7% cellobiose from Avicel™, but 11.48% of cellobiose from bagasse. The addition of gluconic acid yielded 21.9% cellobiose from Avicel™, but 9.2% cellobiose from bagasse, and the addition of gluconolactone produced 31.3% cellobiose from Avicel™ and 11.0% cellobiose from bagasse. Lignocellulosic materials like sugarcane bagasse consist of rigid cellulose fibers embedded in a cross-linked matrix of lignin and hemicelluloses that bind the fibers. Even though it was pretreated with chemicals like ammonia, acids, and lime, it is still complex, and many undesired components-remnant lignin and hemicelluloses and phenolic compounds that might interfere with this inhibition system remain. Studies on the production of cellobiose have been reported by several researchers (Tanaka, 2000; Homma et al., 1993; Tanaka and Oi, 1985). Tanaka (2000) produced 3-3.5 g of cellobiose from 50 g of microcrystalline cellulose by their cellulase component, PEAK I during the
steady state from 6.8-24 days. According to Homma et al. (1993), using cellulase enzyme which had β-glucosidase removed obtained 0.9 g of cellobiose from 46.6 g of cellulose after carrying out repeated batch reaction each for 20 hours three times for a total 60 hours. However, they should perform several enzyme preparations including affinity precipitation, pH control, and fractionation by gel chromatography before enzyme reaction for cellobiose production. Tanaka and Oi (1985) reported that 21 mg of cellobiose was polymerized from 30 g of glucose by β-glucosidase in a three-day incubation. Compared with results from others (Tanaka, 2000; Homma et al., 1993; Tanaka and Oi, 1985), our enzymatic modification system, adding a β-glucosidase inhibitor to a cellulase hydrolysis reaction has potential to be an alternate model to produce a considerable amount of cellobiose. If there is a market for the material, it potentially could be a supplemental product for a biorefinery.

1.5. REFERENCES


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CHAPTER 2. CELLOBIO-OLIGOSACCHARIDE PRODUCTION BY A DEXTRANSUCRASE
CATALYZED REACTION BETWEEN SUCROSE AND CELLOBIOSE

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2.1. INTRODUCTION

Oligosaccharides are carbohydrate polymers, generally of two to ten monomeric residues linked by $O$-glycosidic bonds (Staněk, 1965). Most commercial oligosaccharides were originally developed as sweeteners, but they are currently valued as soluble fibers, which decrease gastrointestinal transit time and moderate constipation and diarrhea. Oligosaccharides are considered to be a low calorie food, because they are resistant to attack by digestive enzymes in humans and animals and are not absorbed by the host (Farworth, 2001). Oligosaccharides may be produced through hydrolysis of polysaccharides, enzymatic synthesis, or extraction from naturally occurring sources. The major commercial oligosaccharides include cyclomaltodextrins, maltodextrins, fructooligosaccharides, galactooligosaccharides, and soy oligosaccharides (Eggleston and Côte, 2003).

Enzymatic production of oligosaccharides has many advantages over other methods, especially chemical methods (Park et al., 1998). Chemical reactions require complicated procedures for the specific protection of hydroxyl groups, whereas this is not necessary for enzymatic processes. An enzyme may transfer one or two hydroxyl groups with the transfer to one being preferred over the other. This limits the reaction steps, simplifies purification and may give higher yields of the desired oligomers. In most cases, the enzymatic synthesis of oligosaccharides has used transglycosylation reactions between a specific donor and a relatively large variety of structurally different acceptors. The configuration of the glycosidic bond produced is a function of the specificity of the transfer by the specific enzyme (Fu et al., 1990; Robyt, 1995). Various oligosaccharides have been produced by enzymatic transfer reactions from glucansucrases (Fu et al., 1990; Robyt 1995), amylosucrases (Skov et al., 2001), cyclodextrin glucanosyltransferases (Vetter et al., 1992; Baek et al., 2000), and sialy transferases (Venot et al., 1994).

Dextransucrase (EC 2.4.5.1) is a glucosyltransferase, which catalyses the transfer of D-glucopyranosyl residues from sucrose to dextran (Monsan et al., 2001). It catalyzes synthesis of a
dextran containing 50% or more α-1,6 glucosidic bonds in the main chain. However, in the presence of an alternate efficient acceptor molecule, its action changes to produce oligosaccharides.

Cellobiose is a disaccharide composed of two glucose molecules linked with a β-1,4 bond, produced during the enzymatic hydrolysis of cellulose. There are some reports on a new class of sugars, containing cellobiose as a component, produced by transglycosylation reactions (Lee et al., 2001; Morales et al., 2001). Acarbose analogues containing cellobiose were prepared by the reaction of acarbose and cellobiose with Bacillus stearothermophilus alpha maltogenic amylase (EC 3.2.1.133; Lee et al., 2001). Cellobiose-acarbose analogues show a potential for use as an inhibitor of β-glucosidase, whereas acarbose does not (Lee et al., 2001). Morales et al. (2001) produced oligosaccharides with branched chains, using cellobiose as an acceptor, in a reaction catalyzed by alternansucrase from Leuconostoc mesenteroides NRRL B-23192. L. mesenteroides B512 FMCM produces an extracellular dextran sucrose which synthesizes a dextran that has 95% α-(1→6) linear and 5% α-(1→3) branched linkages, and can transfer glucosyl units from sucrose onto an acceptor to produce oligosaccharides (Lindberg and Svensson, 1968).

2.2. LITERATURE REVIEW

2.2.1. Enzymatic Synthesis of Oligosaccharides

A wide variety of oligosaccharides have been synthesized by enzymatic transfer reactions between a segment of a specific donor and various acceptors (Park et al., 1998). The enzyme determines the specificity of the transfer and the configuration of glycosidic bond formed. The chemical structure of acceptor is very important in determining the position of transfer for glycosidic bond formation (Fu et al., 1990; Robyt, 1995). Many oligosaccharides have been synthesized by enzymatic transfer reactions involving glucansucrases (Fu et al., 1990; Robyt 1995), alpha amylases (Tonozuka et al., 1994), amylosucrases (Skov et al., 2001), cyclodextrin glucanoyltransferases (Vetter et al., 1992; Baek et al.,
2000), and sialy transferases (Venot et al., 1994). Tonozuka et al. (1994) reported a transglycosylation reaction by *Thermoactinomyces vulgaris* R-47 alpha-amylase. The alpha amylase primarily hydrolyzes α-(1→4)-linked glucose residues in the acceptor molecule. After all the α-(1→4)-linked glucose residues are hydrolyzed, the alpha amylase links glucose to the acceptor molecule by α-(1→3) and α-(1→6) glycosidic linkages. Maltogenic amylase from *Bacillus stearothermophilus* cleaves the first α-(1→4) glucosidic linkage of acarbose to produce glucose and a pseudotrisaccharide (Baek et al., 2000). The pseudotrisaccharide can be transferred to acceptors forming α-(1→6) linkages, followed by α-(1→3) and α-(1→4) glucosidic linkages. Amylosucrase catalyzes the synthesis of an amylase-like polysaccharide composed of only α-(1→4)-glycosidic linkages from sucrose in the presence of glycogen (Skov et al., 2001). Cyclodextrin glucanosyl transferases transfer a maltohexaosyl residue from cyclomaltodextrin to OH-4 of the acceptor molecule to form linear or branched glucooligosaccharides (Vetter, 1992).

### 2.2.2. Dextranucrase

Dextranucrase (1,6-α-D-glucan-6-α-glucosyltransferase, EC 2.4.5.1) catalyzes the synthesis of high molecular weight glucans from sucrose, which contain 50% or more α-1,6 glucosidic bonds as a main chain (Monsan et al., 2001). They are produced by *Leuconostoc* and *Streptococcus* species, as well as by other lactic acid bacteria such as *Lactococci* and *Lactobacilli* (Sidebotham, 1974; Monchois et al., 1999; Monsan et al., 2001). Dextranucrase utilizes sucrose as a high energy donor of glucose instead of using a nucleotide diphospho sugar to drive the reaction. Sucrose induces a change in protein conformation that activates the catalytic site (Mooser, 1992). The energy in the gluco-fructo glycosidic linkage is the same as the energy of a sugar-phosphate linkage in the nucleotide diphospho sugars about 5 kcal/mole (Robyt, 1998). The properties of dextranucrase, produced from *L. mesenteroides* are affected by the strain. *L. mesenteroides* B-512 produces an extracellular dextranucrase that synthesizes
soluble linear α-1,6-linked dextran with about 5% randomly distributed α-1,3-branched linkages containing up to 50 to 100 residues (Robyt and Walseth, 1979). *L. mesenteroides* B-1299 expresses both extracellular and intracellular dextranucrases, synthesizing two types of dextran-fraction L containing 27% of α-1,2- and 1% of α-1,3-branches and fraction S containing 35% of α-1,2 branches. *L. mesenteroides* B-742 generates two dextranucrases that produce a fraction S with 50% α-1,3 branched linkages, and no α-1,4-branch linkages, and a fraction L with 14% α-1,4- and about 1% α-1,3-branched linkages (Kim and Robyt, 1995b). Furthermore, they catalyze the transfer of glucose from sucrose onto an acceptor molecule, synthesizing an oligosaccharide in the presence of an acceptor such as maltose or isomaltose (Koepsell et al., 1953). Figure 2.1 illustrates dextranucrase catalyzed reactions for dextran and oligosaccharides.

Dextranucrase appears to have a molecular mass of 158-195 kDa, a pH optimum of 5.0-5.5, and an optimum temperature of 28-33°C (Naessens et al., 2005; Kim and Robyt 1994b). Low concentrations of calcium are necessary for optimal enzyme production (Robyt and Walseth, 1979). The addition of Tween 80 stabilizes enzyme activity (1994b). Dextranucrase inhibition by 6,6’-dithiodisucrose showed a mixed type inhibition pattern (Eklund and Robyt, 1988) while 6-deoxysucrose, 6-thiosucrose, and α-methyl-D-glucoside showed competitive inhibition (Binder and Robyt, 1985), and 3-deoxysucrose, 4-deoxysucrose, and 4-chloro-4-deoxy-galactosucrose were noncompetitive inhibitors (Tanriseven and Robyt, 1989). Kim et al. (1998) reported that an acarbose effectively inhibited dextran synthesis by dextranucrase by a noncompetitive mechanism. The inhibition was caused by a disproportionation reaction of dextranucrase with isomaltotriose and a decreased efficiency of the maltose acceptor reaction.

**2.2.2.1. Dextran Synthesis**

Dextran is a high molecular weight homo polymer of α-D-glucopyranose units coupled with
Figure 2.1. Reaction catalyzed by dextansucrase. I, Glucan synthesis by successive transfer of glucosyl units; II, sucrose hydrolysis by transfer of the glucosyl unit onto water; III, oligosaccharide synthesis by transfer of the glucosyl unit onto an acceptor molecule; and IV, isotopic exchange by reverse reaction of glucosyl-enzyme complex formation. (Monchois et al., 1999)
main α-1,6 linkages and sometimes branched with α-1,2, α-1,3, or α-1,4 linkages. It has been widely used as a blood volume expander, drug carrier, and chromatographic media due to its non-ionic character and good stability (Koepsell et al., 1952; Shamala and Prasad, 1995). It shows structural variation in the position of the branch linkages, the degree of branching, the length of branch chains, and molecular weight distribution and properties like the degree of solubility in water, depending on the microbial strains that produce it, and according to growth rate and reaction conditions (Kim and Robyt, 1995; Kim et al., 2001; Padmanabhan and Kim, 2002).

2.2.2.1.1. Mechanism for Dextran Synthesis

Dextran is synthesized by autopolymerisation utilizing a single chain mechanism by a unique type of enzyme. Dextran produced at a low rate of the reaction, is high molecular weight (Bovey, 1959; Tsuchiya et al., 1953). Tsuchiya et al. (1953) proposed a mechanism where glucan synthesis may be separated into three different steps—initiation, elongation, and termination.

(1) Initiation of the Reaction

Some researchers have been reported that the addition of exogeneous glucan as a primer helps the polymerization start glucan synthesis (Kobayashi et al., 1986; Germaine et al., 1974 and 1977). In another view, Robyt and Corrigan (1977) negated the necessity of a primer for the initiation of glucan synthesis, presenting that glucansucrases are active enzymes in the absence of any exogenous primer. A dextran itself remained a strong activator of glucan synthesis as it presents blocked hydroxyl groups linked to C6 of the glucosyl residue at its non-reducing end. Also, Robyt et al. (1995) and Mooser (1992) proposed that the reaction may be initiated by conformational change by binding of glucan to enzyme in the catalytic site.

(2) Elongation step

The mechanism of autopolymerization and direction of chain growth are not yet fully
understood (Monchois et al., 1999).

a. Elongation occurs at the non-reducing end of the glucan chain

A carboxyl group, in the presence of an aspartic or glutamic acid, may make a nucleophilic attack on C\textsubscript{1} of the glucosyl moiety of sucrose, driving the formation of a covalent glucosyl-enzyme complex, whereas other acidic groups may facilitate the release of fructose by donating a proton to the oxygen atom in the glucosidic link (Sinnot, 1990). A glucosyl residue can be activated by trapping the hydrogen from the hydroxyl group linked to C\textsubscript{6}. Therefore, only one covalent glucosyl-enzyme complex is required if glucan biosynthesis follows this mechanism. However, Su and Robyt (1994) disagreed with this mechanism because it requires the presence of a primer initiating the glucan biosynthesis and seems not to need sucrose in the chain initiation.

b. Elongation occurs at the reducing end of the glucan chain

Robyt et al. (1974) have proposed a mechanism where elongation occurs at the reducing end of the glucan chain. Dextranucrase may form an enzymatically active covalent complex with glucose and dextran and then glucose is inserted between the enzyme and dextran by a nucleophilic attack of the C\textsubscript{6}-OH of glucose onto C\textsubscript{1} of the dextran forming an α-1→6 glucosidic bond. The released nucleophile attacks another sucrose molecule forming a new enzyme glucosyl intermediate. The C-6 OH group of this new glucosyl intermediate attacks C-1 of dextran, which is actually transferred to the glucosyl residue (Robyt and Eklund, 1983). The glucosyl (dextranosyl) units are alternatively transferred between the two nucleophiles as the dextran chain is elongated at the reducing end. Dextran is built up by extrusion from the enzyme when glucose units are transferred from sucrose to the active site and inserted between the enzyme and the reducing end of the dextran polymer. An additional requirement for this reaction is the transfer of a hydrogen ion to the displaced fructosyl moiety of sucrose. Two imidazolium groups of histidine were required to synthesize dextan. These two imidazolium groups gave
their hydrogen ions to fructose units left behind and resulting imidazole group becomes reprotonated by abstracting a proton from attacking C-6 OH group of the glucosyl-enzyme intermediate, facilitating the nucleophilic attack and the formation of α-1→6 linkages. This mechanism does not require exogenous primer at the beginning of the reaction for a glucan elongation. The mechanism of action of the enzyme from *L. mesenteroides* NRRL B-512F supports this mechanism, showing the presence of two sucrose binding sites (Su and Robyt, 1994).

### 2.2.2.2. Oligosaccharide Synthesis

The acceptor reaction has many possible technical applications for synthesizing oligosaccharide derivatives. Koepsell et al. (1953) found that the dextranucrase changed its pathway for dextran synthesis to oligosaccharide synthesis in the presence of a sucrose medium containing maltose, isomaltose, or *O*-α-methylglucoside. It has been reported that various mono-, di-, tri-, and oligosaccharides acted as acceptors for dextranucrase (Robyt, 1995; Robyt and Eklund, 1983; Fu and Roby, 1990). According to Robyt (1983), maltose and isomaltose are classified as strong acceptors, leucrose and sucrose itself do not seem to be able to act as acceptors. Yoon and Robyt (2002) synthesized new types of 4-maltohexaosyl acarbose and 4-maltododecanosyl acarbose.

Demuth et al. (2002) showed that the synthetic potential for dextranucrase is not restricted to normal saccharides. They tried to use rather unconventional acceptor compounds-alditols, aldulososes, sugar acids, alkyl saccharides, glycols, fructose dianhydride- as an acceptor molecule and sucrose as a donor molecule for transglycosylation by dextranucrase.

In the acceptor reaction of dextranucrase, the glucosyl group from sucrose is successively transferred to the non reducing end of an acceptor molecule making an oligosaccharide that can be alternatively product and substrate. However, other acceptors like fructose produce only leucrose (a disaccharide) which end glucan synthesis (Stolada et al., 1956).
2.2.2.1. Mechanism for Acceptor Reaction

According to their ability to compete with glucan synthesis, or their effect on the reaction velocity acceptor reactions may be separated into two classes: (1) strong acceptors like maltose or isomaltose which are strong inhibitors of glucan synthesis and activators of the reaction velocity and (2) weak acceptors like fructose or meliobiose which are weak inhibitors and activators (Robyt and Eklund, 1983).

Robyt and Walseth (1978) proposed that the acceptor reaction requires a single covalent glucosyl-enzyme complex and it is incorporated at the reducing end of the glucan or the oligosaccharide, producing elongation of the oligosaccharide at the reducing end. Oligosaccharides may be synthesized by a nucleophilic attack of the hydroxyl group located at the non-reducing end of the acceptor to the C1 of one of the two glucosyl residues involved in the two covalent glucosyl-enzyme complexes.

Emeline et al. (2005) reported that the acceptor binding site is unique and consists of the two active catalytic domains, CD1 and CD2. The CD1 is proposed to be involved in the formation of mainly α-1,6 glucooligosaccharides and the CD2 in the synthesis of very few α-1,2 glucooligosaccharides. The study using dsrE gene from L. mesenterides NRRL B-1299 found that CD1 possesses a higher catalytic efficiency than CD2 in the complete construct. Active domains of both CD1 and CD2 can act independently, but alone each shows very low activity.

2.2.2.3. Substrate Specificity of Dextranucrase

Dextran synthesis absolutely requires sucrose, not glucose, mixtures of glucose and fructose or any other sugar (Robyt, 1985). The dextranucrase utilizes the relatively high energy (16.7-20.9 kJ mol⁻¹) of the acetal-ketal linkage joining the glucose and fructose moieties of sucrose in order to synthesize the α-1,6-linkages of the main chain. For this reaction, there are no requirements of ATP or cofactors (Leathers, 2002). Even though sucrose is the only naturally occurring substrate, α-D-glucopyranosyl
fluoride and \( p \)-nitrophenyl-\( \alpha \)-D-glucopyranoside can be substrate but have rates much lower than sucrose (Robyt, 1985).

### 2.2.2.4. DextranSucrase from *Leuconostoc mesenteroides* B512 FMCM

*L. mesenteroides* NRRL B512 (ATCC 10803) produces a single dextranSucrase and synthesizes a wide variety of oligosaccharides by glucosyl transfer reactions to acceptors (Robyt and Walseth, 1978; Robyt and Ekland, 1983). *L. mesenteroides* NRRL B-512 F is the most extensively studied and commercially important strain (Robyt and Walseth, 1979; Santos et al., 2000). *L. mesenteroides* requires sucrose in the medium for the production of dextranSucrase with the exception of constitutive enzyme mutants-B-512 FMC, B-742, B-1142, B-1299, and B1355 (Cote et al., 1999; Kim and Robyt, 1995a; Kim and Robyt, 1995b; Kitaoka and Robyt, 1998; Monsan et al., 2001; Robyt and Walseth, 1979). The growth of *L. mesenteroides* NRRL B512 F is considered to be positively affected by oxygen even though it is a micro-aerophilic strain (Barker and Ajongwen, 1991; Landon and Webb, 1990; Plihon et al., 1995; Lebrun et al., 1994).

The dextranSucrase from *L. mesenteroides* B512 F synthesizes a dextran that has 95% \( \alpha-(1 \rightarrow 6) \) and 5% \( \alpha-(1 \rightarrow 3) \) branch linkages (Lindberg and Svensson, 1968). B-512F is the parent of B512 FM, a hyper producer of dextranSucrase, a mutant produced by nitrosoguanidine (Miller and Robyt, 1984). However, *L. mesenteroides* B512 F dextranSucrase is only produced on induction by sucrose and with low yields. Consequently, the crude enzyme contains a great deal of dextran (Lindberg and Svensson, 1968; Robyt and Walseth, 1979). To overcome these problems, Kim and Robyt (1994) developed *L. mesenteroides* B512 FMC, a constitutive mutant produced by ethyl methane sulfonate mutagenesis. Also, B512 FMC was mutated to B512 FMCM by irradiation with photons (Kim and Kim, 1999). All strains derived from *L. mesenteroides* NRRL B512 F produced the same type of dextran, whereas their activities were different (Kim and Robyt, 1994; Kim and Kim, 1999). B512 FMCM produced 13-times
more enzyme than the parent mutant strain, B512 FMC, and over a 100-fold more than the original B-512F, commercial strain (Kim and Kim, 1999) on sucrose media.

In the acceptor reaction of dextranucrase from L. mesenteroides B-512F, the acceptors are releasing the glucopyranosyl and dextranyl covalent enzyme-intermediates by a nucleophilic displacement reaction, which terminates the polymerization of dextran (Robyt and Walseth, 1978). Su and Robyt confirmed this mechanism by showing that the active site of B-512FM dextranucrase had a single acceptor binding site where the acceptors were bound and could make a nucleophilic attack on the glucopyranosyl or dextranyl group to release them from the active site and to form the acceptor products.

This work reports on optimization of some of the variables that play important roles for the production of cellobio-oligosaccharides; they are temperature, pH, enzymatic concentration, and the concentrations of sucrose and cellobiose. The cellobio-oligosaccharides were purified and their structures were determined.

2.3. MATERIALS AND METHODS

2.3.1. Growth and Culture Conditions

Leuconostoc mesenteroides B-512 FMCM, a constitutive mutant for dextranucrase production, was kindly donated by Dr. Doman Kim (Chonnam National University; Kim and Kim, 1999). The culture was maintained at 30°C in LM medium [0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 2% (w/v) K₂HPO₄, 0.02% (w/v) MgSO₄·7H₂O, 0.001% (w/v) NaCl, 0.001% (w/v) FeSO₄·7H₂O, 0.001% (w/v) MnSO₄·H₂O, 0.013% (w/v) CaCl₂·2H₂O] containing 2% glucose or 2% sucrose. It was maintained on glucose-LM medium containing 2% glucose and 1.5% agar at 4°C, and was transferred biweekly. For growth measurement, samples of 5 ml were taken at desired times for 48 hours. Bacterial growth was measured at 660 nm in a spectrophotometer using 1cm optical cuvettes and the pHs were measured directly.
2.3.2. Enzyme Production

*L. mesenteroides* B-512 FMCM was subcultured by three successive transfers including 1 ml sucrose-LM and glucose-LM medium, 40 ml, and 1 L glucose-LM media to build sufficient volume for inoculation of the final fermentation. The inoculums were 2-5% (v/v) with cultures grown for 16 hours at 30˚C with shaking at 150 rpm. For dextranulcrase production, a 400 ml culture was inoculated to 14 L of LM medium containing 2% glucose and incubated for 48 hours at 30˚C. The pH and agitation were not controlled during fermentation. After harvesting, cells were removed by centrifugation at 6,000 rpm x g for 30 minutes. The cell free culture was concentrated 10-fold using membrane filtration (100K cut off) and washed with 2 volumes of 20 mM sodium citrate buffer, pH 5.2. Tween 80 and NaN₃ were added at concentrations of 1 mg/ml and 0.2 mg/ml to enzyme solution.

2.3.3. Dextranulcrase Assay

Dextranulcrase activity was determined by incubating the enzyme with 100 mM sucrose in 20 mM sodium citrate buffer, pH 5.2 for 1 hour at 30˚C and then boiling for 5 minutes to terminate the enzyme reaction. One unit of dextranulcrase activity was defined as the amount of enzyme releasing 1 µM fructose per minutes from 100 mM sucrose. The fructose was determined by HPLC. Also, relative fructose concentration was measured by TLC. The methods for HPLC and TLC are described below.

2.3.4. Enzyme Characterization

Protein assay was determined using Bio-rad™ protein assay kits following the method of Bradford (Bradford, 1976). Enzyme size was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In order to determine optimal pH and temperature, reaction digests consisted of 100 mM sucrose, 100 mM cellobiose, and 27 U dextranulcrase were incubated for 24 hours under various conditions-pHs ranging from 3.2 to 6.0 and temperatures from 20-50˚C. Transglycosylation products were measured by HPLC.
2.3.5. Box-Behnken Experimental Design

A three-factor and three-level Box-Behnken design was applied for the optimization procedure using Design Expert 6 software (Stat-Ease, Inc.). Several factors—the amounts of sucrose, cellobiose, and dextranulactase—used to prepare each of the 17 formulations are given in Tables 2.1 and 2.2. These high, medium, and low levels were selected from the preliminary experimentation. Optimization was performed using a desirability function to obtain the levels of $X_1$, $X_2$, and $X_3$.

The behavior of the system has been explained by the following quadratic model equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

where $Y$ is the predicted response, $\beta_0$ is the intercept, $\beta_1$, $\beta_2$, and $\beta_3$ are the linear coefficients, $\beta_{11}$, $\beta_{22}$, and $\beta_{33}$ are the squared coefficients and $\beta_{12}$, $\beta_{13}$, and $\beta_{23}$ are the interaction coefficients. A total of 17 experiments were necessary to study the 10 coefficients of the model.

2.3.6. Thin Layer Chromatography

The fructose for dextranulactase activity and oligosaccharides were analyzed by using thin layer chromatography (TLC). The TLC samples were loaded onto a Whatman K5 silica gel plate. For determination of fructose concentration, the plate was irrigated twice with 85% (v/v) acetonitrile. For oligosaccharides, it was irrigated three times with 2:5:1.5 volume parts of nitromethane-1-propanol-water. The carbohydrates on the TLC plate were visualized by dipping the plate into a methanol solution containing 0.3% (w/v) N-(1-naphthyl) ethylenediamine and 5% (v/v) sulfuric acid, followed by heating at 110°C for 15 minutes. The relative percent of carbohydrates was determined using Scion image analyzer software.

2.3.7. High Performance Liquid Chromatography

Concentrations of fructose and crude oligosaccharides were measured by high performance liquid chromatography (HPLC) using an Aminex HPX 87K column (300 mm x 7.8 mm) and HPLC
Table 2.1. Variables in Box-Behnken design

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Levels</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Low</td>
</tr>
<tr>
<td>$X_1$: sucrose (mM)</td>
<td>100</td>
</tr>
<tr>
<td>$X_2$: cellobiose (mM)</td>
<td>50</td>
</tr>
<tr>
<td>$X_3$: dextranucrase (U)</td>
<td>27</td>
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</table>

Table 2.2. Formulations for Box-Behnken design

<table>
<thead>
<tr>
<th>Run</th>
<th>$X_1$</th>
<th>$X_2$</th>
<th>$X_3$</th>
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<td>17</td>
<td>400</td>
<td>250</td>
<td>40</td>
</tr>
</tbody>
</table>
analyzer coupled to a refractive index detector. The column was maintained at 85°C and 0.01 M K₂SO₄ was used as a mobile phase at a flow rate, 0.6 ml/min.

2.3.8. High Performance Anion Exchange Chromatography

The enzyme reaction products were analyzed by high performance anion exchange chromatography (HPAEC) using a Dionex Carbo-Pac PA 100 column (250 x 4 mm) by gradient elution using 1 M NaOH, water, and 480 mM sodium acetate at a constant flow rate of 0.5 mL/min. Oligosaccharide detection was carried out with an electrochemical detector (ED 40).

2.3.9. Production of Cellbio-oligosaccharides

Transglycosylation reactions were performed in 500 ml of 20 mM citrate buffer (pH 5.2) including 300 mM of sucrose, 250 mM of celllobioe, 54 U dextranase at 30°C with shaking at 150 rpm. The reaction was performed until the sucrose was depleted and then terminated by heating for 20 minutes at 95°C. A reaction product was centrifuged at 6,500 rpm for 45 minutes for the removal of insoluble polysaccharide. The soluble polysaccharide was precipitated with an equal volume of ethanol which was slowly added to the supernatant and the resulting solution stored in a refrigerator for 2 hours. The precipitate was eliminated by centrifugation at 6,500 rpm for 45 minutes. The supernatant was analyzed by HPLC, TLC, and HPAEC as described above. The supernatant was concentrated 10-fold using a rotary evaporator and then freeze dried.

2.3.10. Purification of Cellbio-oligosaccharides

The crude oligosaccharide solution was loaded onto Bio-Gel P2 (fine) column (1.5 cm x 115 cm), and eluted with water. It was collected in 0.5-1.0 ml fractions. Their purities were analyzed using either TLC or HPAEC mentioned above. Those fractions with the same DP were pooled and freeze dried.

2.3.11. Liquid Chromatography-Mass Spectrometry

Mass spectrometry data of the purified oligosaccharides were obtained from electrospray (MS-
ES) measurements. The solvent was ultrapure water at 7 µl/min\(^{-1}\) and detection was performed in the positive mode.

### 2.3.12. Nuclear Magnetic Resonance

About 50 mg from the purified oligosaccharides were exchanged three to four times with 600 µl of pure D\(_2\)O and lyophilized twice, and then dissolved in 600 µl of pure D\(_2\)O, and placed into NMR tubes. NMR spectra were produced using a spectrometer, operating at 500 MHz for \(^1\)H and 125 MHz for \(^{13}\)C at 25°C. It was examined for the linkages between cellobiose and glucose from homonuclear correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), rotating frame overhause effect spectroscopy (ROESY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple quantum coherence spectroscopy (HMQC) spectra.

### 2.3.RESULTS AND DISCUSSION

#### 2.4.1. Production of Dextranucrase

*L. mesenteroides* B-512 FMCM, a mutant strain of *L. mesenteroides* B-512, produced dextranucrase without any dextran contamination on glucose-LM medium. Its growth, production of dextranucrase, and pH changes of media were examined (Figure 2.2). The maximum cell concentration (OD \(_{660 nm}\) 1.18) was obtained at the 27th hour. According to Cortezi et al. (2005), extracellular dextranucrase production was closely related to bacterial growth when 3-4% sucrose was used as a carbon source. We found that the production of dextranucrase was commenced 12 hours after growth started. The highest enzyme activity, 0.332 U/mg protein, was found in the late stationary phase between 30 and 44 hours. Production and activity of enzyme seemed not to be affected as the pH of medium dropped below 4.8, a beyond pH range some researchers (Alsop, 1983; Chen and Kaoli, 1976) reported. *L. mesenteroides* grown on sucrose medium as a carbon source has been reported to produce maximum dextranucrase at pH 6-7. Tsuchiya et al. (1952) found that dextranucrase production is optimal at pH
Figure 2.2. The growth of *L. mesenteroides* B-512 FMCM, pH changes, and the production of dextranucrase. Cells were incubated for the production of dextranucrase in a LM medium containing 2% glucose at 30°C. All samples were collected with time intervals, and directly measured for cell growth and pH changes, stored at 0°C for dextranucrase activity assay. For dextranucrase activity, all samples were incubated with 100 mM sucrose at 30°C for 1 h and fructose were measured using TLC at the same time. Dextranucrase activity was indicated as their relative activity which is the % of fructose concentration for test dextranucrase sample to the highest fructose concentration among all collected samples.
values between 7 and 8, but the enzyme denatured irreversibly when maintained at that pH range for an extended period of time. However, Padmanabhan and Kim (1992) produced dextranucrase at approximately pH 5.

2.4.2. Enzyme Characterization

The concentrated dextranucrase used for transglycosylation reaction had a specific activity of 22.15 U/mg total protein. Its size was 180 kDa by SDS-PAGE after Rf values were calculated using distances of bands in a standard marker and dextranucrase (Figure 2.3). The filtration step using a 100 kDa membrane filter effectively removed sucrose catalyzed enzymes; the 65 kDa isomerase and the 55 kDa sucrose phosphorylase. The dextranucrase occurs in multiple molecular forms due to enzyme aggregation (Robyt and Walseth, 1979).

The optimum pH for cellobio-oligosaccharide product ion was 5.2 (Figure 2.4). The optimum pH for oligosaccharide production was the same as that for dextranucrase activity (Kim and Robyt, 1994a). The production of cellobio-oligosaccharides was more sensitive to the changes in pH than was dextranucrase activity. Dextranucrase produced 39% cellobio-oligosaccharide at pH 6.2 comparing to 100% cellobio-oligosaccharide production at pH 5.2.

There was a sharp decrease in the production of cellobio-oligosaccharides by dextranucrase above 30°C (Figure 2.5). It only transglycosylated 46 % of the glucosyl units from sucrose to cellobiose of the maximum at 40°C. Dextranucrase for oligosaccharide production has the same pattern as the effect of temperature of dextranucrase activity on temperature changes (Kim and Robyt, 1994a).

2.4.3. Box-Behnken Design

Table 2.3 depicts the actual and predicted values of cellobio-oligosaccharides on the basis of the experimental design (Kim and Day, 2007). The predicted values and the observed values agreed reasonably well. Concerning the p-value of the coefficients, $X_1$, $X_2$, $X_3$, and $X_1^2$ were found to have
Figure 2.3. SDS-PAGE of dextranulcase of *L. mesenteroides* B-512 FMCM. Standard means a standard marker (175 kDa of MBP-β-galactosidase from *E. coli*, 83 kDa of MBP-paraomyosin from *E. coli*, 62 kDa of glutamic dehydrogenase from bovine liver, 47.5 kDa of aldolase from rabbit muscle, 32.5 kDa trisephosphate isomerase from *E. coli*, 25 kDa of CBD-BmFKBP13 from *E. coli*, 16.5 kDa of lysosyme from chicken egg white, and 6.5 kDa of aprotinin from bovine lung; New England BioLabs Inc.). A is a crude dextranulcase after 30 K membrane filtered. B is a dextranulcase after 100 K membrane filtered.
Figure 2.4. Production of cellobio-oligosaccharides synthesized by *L. mesenteroides* B-512 FMCM dextranulcrase at various pH values.

1Relative activity for cellobio-oligosaccharide production was calculated as the percentage concentration of cellobio-oligosaccharides at the selective pH, divided by the highest concentration of cellobio-oligosaccharides over all pH ranges.

2Reactions conducted at 100 mM sucrose, 100 mM cellobiose, dextranulcrase 27 U, and 30°C for 24 h.
Relative activity for cellobio-oligosaccharide production was calculated as the percentage concentration of cellobio-oligosaccharides at the selective temperature, divided by the highest concentration of cellobio-oligosaccharides over all temperature ranges.

Reactions conducted with 100 mM sucrose, 100 mM cellobiose, dextranucrase 27 U, and 30°C for 24 h.
Table 2.3. Observed and predicted values and variance parameters of cellobio-oligosaccharide yield (%) recorded in experimental set up of response surface methodology

<table>
<thead>
<tr>
<th>Run #</th>
<th>Observed Y</th>
<th>Predicted Y</th>
<th>Residuals</th>
</tr>
</thead>
<tbody>
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<td>33.60</td>
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<td>2.77</td>
</tr>
<tr>
<td>2</td>
<td>35.72</td>
<td>36.37</td>
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</tr>
<tr>
<td>3</td>
<td>63.25</td>
<td>65.39</td>
<td>-2.15</td>
</tr>
<tr>
<td>4</td>
<td>64.12</td>
<td>64.09</td>
<td>0.02</td>
</tr>
<tr>
<td>5</td>
<td>35.29</td>
<td>37.51</td>
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<td>6</td>
<td>73.67</td>
<td>74.19</td>
<td>-0.52</td>
</tr>
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<td>7</td>
<td>66.78</td>
<td>71.25</td>
<td>-4.47</td>
</tr>
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<td>17</td>
<td>67.86</td>
<td>70.63</td>
<td>-2.77</td>
</tr>
</tbody>
</table>
significant effects on the performance of the model for the prediction of the cellobio-oligosaccharide production (Tables 2.4 and 2.5; Kim and Day, 2007).

The final estimative response model equation was as follows:

\[ Y = 71.91 + 7.64X_1 + 12.36X_2 + 17.82X_3 - 12.98X_1^2 - 3.29X_2^2 - 0.41X_3^2 - 4.25X_1X_2 + 3.31X_1X_3 - 0.52X_2X_3 \]

where \( Y \) is the response factor (cellobio-oligosaccharide production, \%) and \( X_1, X_2 \) and \( X_3 \) represent real values of the independent factors—sucrose (mM), cellobiose (mM) and dextrantrucrase (U). Table 2.4 shows the model coefficients and probability values (Kim and Day, 2007). The ANOVA of this model (Table 2.5; Kim and Day, 2007) demonstrated that the model is highly significant, as is evident from the \( F \) (\( F_{model} = 35.99 \)) and a very low probability value (\(<0.0001\)). The \( P \) value lower than 0.1 indicates that a model is considered to be statistical significant (Kim et al., 2003). Also, \( F \)-value of 0.64 for the lack of fit implies that it is not significant relative to the pure error according to our results of analysis of factors. Non-significant lack of fit is a good indication that the model fits the actual relationships of the reaction parameters within the selected ranges.

The relationship between the independent and dependent variables was elucidated using contour and response surface plots (Figure 2.6; Kim and Day, 2007). Each plot represents the effect of two variables at their studied range with the other one maintained at fixed level. The shapes of contour plots display the nature and extent of the interactions (Fu et al., 2006). As shown in Figure 2.6a, an increase in sucrose concentration up to 300 mM results in increased cellobio-oligosaccharide production with increases in cellobiose concentration at 40.5 U dextrantrucrase. An increase in the concentration of cellobiose causes a linear increase in cellobio-oligosaccharide produced. However, sucrose concentrations greater than 300 mM interfere with cellobio-oligosaccharide production despite increases in the concentrations of cellobiose. The reaction solutions became increasingly viscous with increases in
Table 2.4. Regression coefficients and significance of regression model

<table>
<thead>
<tr>
<th>Factor</th>
<th>Coefficient estimate</th>
<th>DF</th>
<th>Standard error</th>
<th>95% CI low</th>
<th>95% CI high</th>
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<tr>
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<td>X₁</td>
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<td>-1.39</td>
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<td>X₂X₃</td>
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<td>1</td>
<td>1.99</td>
<td>-5.22</td>
<td>4.18</td>
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CI, confidence interval.
Table 2.5. ANOVA for response surface

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<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>DF</th>
<th>Mean square</th>
<th>F-value</th>
<th>Probability &gt;F&lt;sup&gt;a,b&lt;/sup&gt;</th>
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<sup>a</sup>Probability > F, level of significance.

<sup>b</sup>Values of “probability > F” less than 0.05 indicate model terms are significant. Values greater than 0.1 indicate the model terms are not significant.
Figure 2.6. Response surface plots illustrating the effect of varying concentrations of sucrose, cellobiose, dextransucrase on their reciprocal interactions with cellobio-oligosaccharide production. Other factors are held constant at 40.5U of dextranucrase (a); 150 mM of cellobiose (b); 250 mM of sucrose (c). The reactions were performed in 20 mM citrate buffer, pH 5.2 at 30°C and 150 rpm.
sucrose concentration due to the concomitant formation of dextran. This finding is similar to that reported for the action of sucrose on the dextransucrase production by *L. mesenteroides* B-512 F (Goyal and Katiyar, 1997).

The effects of varying dextransucrase and sucrose concentrations on the cellobio-oligosaccharide production with 150 mM celllobiose are shown in Figure 2.6b. Highest production of cellobio-oligosaccharides was achieved at the middle range of sucrose concentration across the entire range of dextransucrase concentration. A linear increase in dextransucrase does not lead to a continual increase of cellobio-oligosaccharide production with increases in sucrose concentration. Product inhibition was observed due to dextran formation above 54 U of dextransucrase (data not shown). Dextransucrase catalyzes dextran synthesis as well as oligosaccharide synthesis. Kim and Robyt (1994b) reported that dextran production showed a slightly sigmoidal increase suggesting an allosteric effect for the dextran.

Figure 2.6c illustrates the effects of different dextransucrase concentrations and celllobiose concentrations on the cellobio-oligosaccharide production in the presence of 250 mM sucrose. Increases in the amount of both dextransucrase and celllobiose resulted in linear increases in the production of cellobio-oligosaccharides when sucrose concentration was fixed. The highest concentrations were reached using high dextransucrase and celllobiose. However, the addition of celllobiose was limited by the solubility of celllobiose. Figure 2.7 suggests that high celllobiose and dextransucrase as well as sucrose concentration between 250 and 300 mM achieve the optimal transglycosylation of sucrose to celllobiose. The optimum values for the selected factors were calculated from the regression equation. The optimal conditions for the cellobio-oligosaccharides were as follows: $X_1 = 288.96$ mM, $X_2 = 250$ mM, $X_3 = 54$ U. The theoretical cellobio-oligosaccharide yield predicted under these conditions was $Y=98.74\%$. The prediction value by the model was confirmed using the above conditions.
Figure 2.7. Response surface plot representing the effect of varying concentrations of sucrose, cellobiose, dextranucrase on their reciprocal interactions with cellobio-oligosaccharide. Dextranucrase is held constant at 54 U. The reactions were performed in 20 mM citrate buffer, pH 5.2 at 30°C and 150 rpm.
2.4.4. Transglycosylation Reaction

Dextranucrase from *L. mesenteroides* B-512FMCM catalyzed an acceptor reaction between cellobiose and sucrose. As shown in Figure 2.8, cellobiose acceptor reaction products display a cellobiose-oligosaccharide peak, whereas dextran synthesis reaction in the absence of cellobiose does not. However, HPLC chromatograms were not capable of separating each oligosaccharide of the cellobio-oligosaccharides. Using HPAEC chromatogram, we obtained one major product (B), several minor products (A, C, D, and E), fructose and leucrose (Figure 2.9). The product A and B had the same position on TLC (Figure 2.9).

The cellobio-oligosaccharide yields were shown in Table 2.6. Transglycosylation reaction between cellobiose and sucrose by *L. mesenteroides* B-512 FMCM dextranucrase produced 10.7 mg/ml cellobio-oligosaccharides. The concentrations were 1.5 mg/ml of A, 5.5 mg/ml of B, 1.4 mg/ml of C, 1.4 mg/ml D, and 0.9 mg/ml of E.

2.4.5. Purification of Cellobio-oligosaccharides

The transglycosylation products were fractionated by Bio-Gel P2 gel permeation chromatography (Figure 2.10). Unreacted cellobiose and glucose, and by-products of transglycosylation, leucrose and fructose were removed from reaction products, A, B, C, D, and E. Each reaction product was purified and separated for structure analysis and experiments on biological activity.

2.4.6. Mass Spectrometry

The numbers of D-glucose units that were reacted with cellobiose for products A, B, C, D, and E were determined by using MALDI-TOF MS. The data for molecular masses were detected in the positive mode and their masses determined after Na\(^+\), mass (22.99 g/mol), was removed. The mass of the product A and B indicated 504.07 g/mol (Figure 2.11a), C 666.02 g/mol (Figure 2.11a), D 828.28 (Figure 2.11b), and E 990.33 (Figure 2.11b). The masses of these reaction products increased over that
Figure 2.8. HPLC of products of transglycosylation reaction. The red line indicates the transglycosylation products with 100 mM sucrose, 100 mM cellobiose, and 27 U dextranucrase in 20 mM citrate buffer, pH 5.2 at 30°C for 24 h. The blue line indicates the transglycosylation products with 100 mM sucrose and 27 U dextranucrase in 20 mM citrate buffer, pH 5.2 at 30°C for 24 h. Aminex HPX 87K column was used and a refractive index detector was used as a detector. Running temperature was 85°C and 0.01 M K$_2$SO$_4$ was used as a mobile phase at a flow rate 0.6 ml/min.
Figure 2.9. HPAEC and TLC of products of transglycosylation reaction. The transglycosylation was conducted in 20 mM citrate buffer (pH 5.2) including 300 mM of sucrose, 250 mM of cellobiose, 54 U dextranucrase at 30°C until sucrose was depleted. All glucan polymers (dextrans) were removed. Dionex Carboxpak PA 100 column was used and electrochemical detector was used, and gradient elution was used 1 M NaOH, water, and 480 mM sodium acetate at a constant flow rate of 0.5 ml/min for HPAEC. The solution for irrigation contained 2:5:1.5 parts of nitromethane: 1-propanol: water for TLC.
Table 2.6. Concentration and apparent yield of cellbio-oligosaccharides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mg/ml)</th>
<th>Apparent yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.5</td>
<td>14.0</td>
</tr>
<tr>
<td>B</td>
<td>5.5</td>
<td>52.7</td>
</tr>
<tr>
<td>C</td>
<td>1.4</td>
<td>12.9</td>
</tr>
<tr>
<td>D</td>
<td>1.4</td>
<td>13.3</td>
</tr>
<tr>
<td>E</td>
<td>0.9</td>
<td>8.1</td>
</tr>
</tbody>
</table>
Figure 2.10. Bio-Gel P2 gel permeation column chromatogram of the *L. mesenteroides* B-512 FMCM dextransucrase reaction products. The column was 1.5 cm x 115 cm and eluted with water, and collected with 0.5 ml fractions.
Figure 2.11a. MALDI-TOF MS analysis of transglycosylation products by the dextransucrase *L. mesenteroides* B-512 FMCM. (A) Products-A and B; (B) product-C.
Figure 2.11b. MALDI-TOF MS analysis of transglycosylation products by the dextranulase \textit{L. mesenteroides} B-512 FMCM. (C) product-D; (D) product-E.
of cellobiose by exactly a single D-glucose residue (M.W. 162 g/mol). Therefore, A and B were 
trisaccharides, C was tetrasaccharide, D was pentasaccharide, and E was a hexasaccharide.

2.4.7. NMR Spectrometry

The structures of the major transglycosylation products A and B were determined by $^1$H and $^{13}$C 
nuclear magnetic resonance (NMR) spectrometry in order to determine the synthetic modes of cellobio-
oligosaccharides. The proton signals were assigned from analyses of $^1$H/$^1$H-COSY and $^1$H/$^1$H-TOCSY 
spectra. After the assignment of all proton signals, the corresponding $^{13}$C resonances were allowed by 
$^1$H/$^{13}$C-HSQC spectrum, followed by ROESY and HMQC. All assignments of the cellobio-
oligosaccharides are shown in Table 2.7.

The NMR assignments indicated two forms of trisaccharides. We noted a smaller amount of 
trisaccharide as a product A and a larger amount of trisaccharide as a product B. When the integral of 
III-1 proton at 5.06 ppm was determined as 1, the total integrals of two III-1 proton at 5.33 and 4.96 ppm 
were 2.2 (Figure 2.12). Therefore, a trisaccharide having III H-1 at 5.06 ppm was determined as a 
product A and the other as a product B.

The new anomeric proton signal at 5.06 ppm (J = 3.5 Hz, doublet signal) was assigned, 
indicating that a glucosyl residue was connected to cellobiose with α-linkage (Agrawal, 1992). In a 
product A, the $^{13}$C-chemical shift in cellobiose before and after the addition of α-D-glucopyranose to 
cellobiose for C-6 was changed from 60.932 ppm to 66.354 ppm (Table 2.7 and Figure 2.13). This 
chemical shift change is characteristic of the attachment of a D-glucopyranose unit to the original 
glucoside or aglycone (Agrawal, 1992; Yoon et al., 2004; Kono et al., 1999). Except for this change for 
C-6, the spectra of a product A gave no resonance changes. Therefore, the NMR result indicates that the 
D-glucopyranose unit was attached to the cellobiose ring by an α-(1→6) linkage. The cellobio-
oligosaccharide structure was proposed in Figure 2.14(a).
Table 2.7. $^1$H NMR and $^{13}$C NMR chemical shifts$^a$ for product A and B produced by the reaction of dextranucrase with sucrose and cellobiose (units: ppm).

<table>
<thead>
<tr>
<th>Cellobiose (δ)</th>
<th>CBO-A$^b$ (δ$_A$)</th>
<th>CBO-B$^c$ (δ$_B$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>δ$_C$</td>
<td>δ$_{AC}$</td>
</tr>
<tr>
<td>I$^d$ α-Glc 1$^e$</td>
<td>92.20</td>
<td>92.33</td>
</tr>
<tr>
<td>2</td>
<td>71.60</td>
<td>71.83</td>
</tr>
<tr>
<td>3</td>
<td>71.71</td>
<td>72.28</td>
</tr>
<tr>
<td>4</td>
<td>79.11</td>
<td>79.03</td>
</tr>
<tr>
<td>5</td>
<td>70.48</td>
<td>70.40</td>
</tr>
<tr>
<td>6</td>
<td>60.28</td>
<td>60.63</td>
</tr>
<tr>
<td>β-Glc 1</td>
<td>96.12</td>
<td>96.54</td>
</tr>
<tr>
<td>2</td>
<td>74.26</td>
<td>73.58</td>
</tr>
<tr>
<td>3</td>
<td>74.66</td>
<td>73.46</td>
</tr>
<tr>
<td>4</td>
<td>78.97</td>
<td>79.07</td>
</tr>
<tr>
<td>5</td>
<td>75.16</td>
<td>73.58</td>
</tr>
<tr>
<td>6</td>
<td>60.42</td>
<td>61.232</td>
</tr>
<tr>
<td>II β-Glc 1</td>
<td>102.93</td>
<td>103.46</td>
</tr>
<tr>
<td>2</td>
<td>73.54</td>
<td>73.30</td>
</tr>
<tr>
<td>3</td>
<td>76.86</td>
<td>76.12</td>
</tr>
<tr>
<td>4</td>
<td>69.82</td>
<td>70.11</td>
</tr>
<tr>
<td>5</td>
<td>76.34</td>
<td>75.32</td>
</tr>
<tr>
<td>6</td>
<td>60.94</td>
<td>66.35</td>
</tr>
<tr>
<td>III α-Glc 1</td>
<td>96.89</td>
<td>5.06</td>
</tr>
<tr>
<td>2</td>
<td>71.74</td>
<td>3.53</td>
</tr>
<tr>
<td>3</td>
<td>73.37</td>
<td>3.77</td>
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<tr>
<td>4</td>
<td>69.82</td>
<td>3.43</td>
</tr>
<tr>
<td>5</td>
<td>70.52</td>
<td>3.95</td>
</tr>
<tr>
<td>6</td>
<td>61.31</td>
<td>3.78</td>
</tr>
</tbody>
</table>

$^a$Chemical shifts were measured at 125 MHz for $^{13}$C NMR and 500 MHz for $^1$H NMR in D$_2$O at 25°C with acetone as an internal standard.

$^b$α-D-glucopyranosyl-(1→6)-cellobiose.

$^c$α-D-glucopyranosyl-(1→2)-cellobiose.

$^d$Each of the residues of celllobio-oligosaccharide is designed by Roman Numerals, started with I at the reducing-end residue.

$^e$The position of carbon and proton and the number starts from the anomeric carbon in a residue.
Figure 2.12. $^1$H NMR spectrum of A [α-D-glucopyranosyl-(1→6)-cellobiose] and B [α-D-glucopyranosyl-(1→2)-cellobiose] in D$_2$O. Chemical shifts were measured at 500 MHz for $^1$H NMR. Each of the residues of carbohydrates is designed by Roman Numerals, started with I at the reducing-end residue.
Figure 2.13. $^{13}$C NMR spectra of (a) cellobiose and (b) $\alpha$-D-glucopyranosyl-(1→6)-cellobiose and $\alpha$-D-glucopyranosyl-(1→2)-cellobiose. Chemical shifts were measured at 125 MHz for $^{13}$C NMR. Each of the residues of carbohydrates is designed by Roman Numerals, started with I at the reducing-end residue.
In a product B, the $^1$H chemical shifts of the new anomeric carbon (C-1) were 5.33 and 4.96 ppm with a coupling constant of 3.5 Hz, indicating that they were α-conformation (Table 2.7 and Figure 2.12; Agrawal, 1992). The corresponding $^{13}$C chemical shifts appeared at 98.29 and 98.59 ppm (Table 2.7 and Figure 2.13). According to Bock et al. (1986), a $^{13}$C chemical shift of C-1 in α-D-glucopyranose-(1→2)-β-D-glucopyranose was 98.6 ppm, indicating an α-(1→2) linkage. Evidence for this linkage was supported by a downfield $^{13}$C shift for II C-2 of cellobiose from 73.54 to 76.73 (Table 2.7 and Figure 2.13). These results identified the cellobio-oligosaccharide structure as α-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-D-glucopyranose (Figure 2.14b).

Our finding was agreed with Morales et al. (2001) who reported that $L$. mesenteroides B-512 F dextranucrase synthesized α-D-(1→2)-glucopyranosyl cellobiose and α-D-(1→6)-glucopyranosyl cellobiose in the presence of cellobiose. Besides these two products, they produced α-D-glucopyranosyl-(1→3)-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→6)-cellobiose and α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→6)-cellobiose when cellobiose was used the acceptor molecule by alternansucrase from $L$. mesenteroides B-23192. However, dextranucrase from $L$. mesenteroides B-512 F is known primarily to transfer the D-glucose residue from sucrose to the non-reducing end 6-hydroxyl group of mono- and higher-saccharides in the presence of an acceptor molecule (Robyt, 1995; Robyt and Eklund, 1983). Interestingly, the synthesis of oligosaccharides by $L$. mesenteroides B-512 F dextranucrase apparently depends on the type of acceptor molecule. In the presence of a β-glucosidic linkages in the acceptor molecule, the specificity of dextranucrase is changed to transfer the 2-OH group at the reducing end glucose rather than transfer 6-OH at the non-reducing end (Yoon and Robyt, 2002; Robyt, 1995; Robyt and Eklund, 1983). It transfers the D-glucose residue to the non-reducing end OH of maltose or isomaltose in the presence of maltose or isomaltose whereas it transfers D-glucose from sucrose to the reducing end D-glucose as well as the 6-OH groups of the non-reducing end in the
Figure 2.14. Proposed chemical structures of (a) α-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-D-glcopyranose and (b) α-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-D-glcopyranose. Each of the residues of cellbio-oligosaccharides is designed by Roman Numerals, started with I at the reducing-end residue.
presence of maltotriose and maltotetraose (Fu and Robyt, 1990). When the acceptor molecule is lactose or raffinose, \textit{L. mesenteroides} B-512 F dextrantransferase transfers D-glucose from sucrose to the OH group at C-2 of the D-glucose residue (Robyt, 1995; Robyt and Eklund, 1983).

\section*{2.5. REFERENCES}


Kim, D.-W., Heo, S.-J., and Ryu, S.-J. Enzyme capable of hydrolyzing plaque, microorganism producing the same, and a composition comprising the same. US patent issued on November 26, 2002.


CHAPTER 3. BIOLOGICAL PROPERTIES OF CELLOBIO-OLIGOSACCHARIDES
3.1. INTRODUCTION

Oligosaccharides have been widely used in food, feed, pharmaceutical, and cosmetic industries due to their beneficial effects in humans and animals (Eggleston and Cote, 2003). There is great interest in various physiological functions of oligosaccharides such as immune-stimulation, anti-cariogenic, and prebiotic effects as well as uses for sweeteners, stabilizers, and bulking agents (Eggleston and Cote, 2003; Otaka, 2006). Most of the beneficial effects related to health have originated from their role as inhibitors of enzymes involved in carbohydrate metabolism.

Dental caries is acid induced decay of the teeth which is mainly caused by colonization by oral streptococci primarily *Streptococcus mutans* and *S. sobrinus* (Michalek and Childers, 1990). Oral streptococci secrete a mutansucrase which synthesizes from sucrose to glucans with a majority of α-(1-3)-glucosidic linkages and a highly branched structure (Wenham et al., 1981; Freedman et al., 1978). α-(1-3)-D-Glucan produced by mutansucrase, called a mutan, is an adherent, and water insoluble polysaccharide (Guggenheim and Newbrun, 1969). Mutans mediate interactions and accumulation of the bacteria to tooth surfaces, such that they induce the development of dental caries (Lee 2005; Rolla et al., 1983). Certain oligosaccharides, but not based on cellobiose, have been reported as preventing the occurrence of dental caries (Otaka, 2006).

The α-glucosidase inhibitors have been utilized in the therapeutic treatment of the metabolic diseases-diabetes and obesity in conjunction with α-amylase (Asano et al., 2000; Watanabe et al., 1997). The α-glucosidase inhibitors can effectively overcome a side effect of α-amylase inhibitors, flatulence by intestinal bacteria caused by undigestible polysaccharides, since α-glucosidase inhibitors catalyze the final step in dietary carbohydrate (Bischoff, 1995; Lee, 2005; Truscheit et al., 1981). α-Glucosidase inhibitors interfere with the conversion of maltose to glucose and suppress postprandial hyperglycemia (Watanabe et al., 1997). An acarbose consisting of acarbosine and maltose linked with α-1,4-glucosidic
linkage is a well known α-glucosidase inhibitor that has been used to reduce postprandial hyperglycemia (Truscheir et al. 1981; Watanabe et al., 1997).

An opportunistic infectious disease, invasive aspergillosis has emerged as a major cause of morbidity and mortality in immunocompromised people (Denning et al., 1998; Lin et al., 2001). Aspergillus fumigates, A. flavus, and A. terreus are the species which cause about 95% of the pathogenic cases (Richardson and Warnock, 2003). Recently, A. terreus infection has emerged as a growing concern because it produces different epidemiological features, a more aggressive clinical behavior, and a much higher mortality rate than infection caused by other Aspergillii (Castón et al., 2007; Steinbach et al., 2004). Mortality has been reported to reach 100% in patients infected by A. terreus. A. terreus is completely resistant to a widely used antifungal drug, amphotericin B (AmB) (Lass-Flörl et al., 1998; Johnson et al., 2000).

1,3-β-D-Glucan synthase (GS) catalyzes the synthesis of 1,3-β-D-glucan, a vital structural polymer of the fungal cell wall that is also a potential antifungal target for therapy of serious fungal infections (Huang et al., 1990; Tkacz, 1992). The 1,3-β-D-glucan of fungal cell is unique, not being found in other eukaryotes. Consequently, GS inhibitors can be excellent antifungal agents with showing a high target specificity, reducing side effects to the host. Echinocandins, pneumocandins, and papulacandins are examples of GS inhibitors, but they each have some drawbacks such as ineffectiveness against some fungal species and insolubility (Kurtz et al., 1994; Latgé, 2007; Hector, 1993).

3.2. LITERATURE REVIEW

3.2.1. Beneficial Health Effects of Oligosaccharides

Table 3.1 summarizes the beneficial health effects of oligosaccharides.
Table 3.1. Summary of various beneficial functions of oligosaccharides

<table>
<thead>
<tr>
<th>Functions</th>
<th>Oligosaccharides</th>
<th>Beneficial health effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indigestibility</td>
<td>Most oligosaccharides except for coupling sugar and isomaltulose(^a)</td>
<td>Low calorie</td>
</tr>
<tr>
<td>Prebiotic</td>
<td>Fructooligosaccharides(^{bcd}), Galactooligosaccharides(^{bce}), Lactulose(^{bc}), Glucoooligosaccharides(^{fg}), Xyloooliosaccharide(^{h}), etc.</td>
<td>Improved intestinal microbiota, Improved tolerance to lactose, Protection from gastroenteritis, Prevention of bowel cancer, Prevention of coronary heart disease, Vitamin synthesis, Decreased carcinogenesis</td>
</tr>
<tr>
<td>Anticariogenesis</td>
<td>Maltooligosylsucrose(^{i}), palatinose(^{i}),Neosugar(^{a}), maltitol(^{a}), lactitol(^{a})</td>
<td>Prevents dental caries</td>
</tr>
<tr>
<td>Anticarcinogenesis</td>
<td>β-oligosaccharides(^{j}), chitosan-oligosaccharides(^{k})</td>
<td>Prevents cancer</td>
</tr>
<tr>
<td>Modulation of immune system</td>
<td>Mannooligosaccharides(^{l})</td>
<td>Enhances immune system with increased IgA and lymphocyte</td>
</tr>
</tbody>
</table>

\(^a\)Oku, 1996; \(^b\)Ziemer and Gibson, 1998; \(^c\)Fuller and Gibson, 1997; \(^d\)Probert et al., 2004; \(^e\)Gopal et al., 2001; \(^f\)Flickinger et al., 2000; \(^g\)Djouzi et al., 1995; \(^h\)Kabel et al., 2002; \(^i\)Ooshima et al., 1983; \(^j\)Hidaka, 1994, \(^k\)Jeon and Kim, 2002; \(^l\)Swanson et al., 2002; \(^m\)Miyanishi et al., 2003.
3.2.1.1. Indigestibility

Most oligosaccharides provide less metabolic energy than sucrose because they are either not or poorly hydrolyzed by digestive enzymes (Oku, 1996). Only selected intestinal bacteria can metabolize nondigestible oligosaccharides (Oku, 1996; Nakamura, 2004). The ability of selected bacteria to use these oligosaccharides impacts other beneficial effects of oligosaccharides. Neosugar, a mixture of fructooligosaccharides such as kestose, nystose, and fructofuranosyl nystose, is not digested in the small intestine but rather reaches the large intestine where it is completely fermented by the intestinal bacteria (Tokunaga et al., 1989; Oku, 1996). When Neosugar was injected intravenously into a rat, it was not hydrolyzed by any enzymes in organs and was not decomposed when it reached the urine (Tokunaga et al., 1989). Orally administered Neosugar postponed the release of CO₂ about 3 hours compared with orally administered sugar (Tokunaga et al., 1989). When Neosugar was incubated anaerobically with the cecal content of rats, short-chain fatty acids (SCFAs) as well as CO₂ were released spontaneously (Tokunaga et al., 1989). Similar observations were made on the metabolism of oligosaccharides such as raffinose, stachyose, lactulose, galactosyl-sucrose, 4’ galactooligosaccharide, 6’galactooligosaccharides, xylooligosacchaides, lacitol, isomaltitol, and maltitol (Oku, 1994). When the combustion energy of SCFA released by intestinal bacteria was used as the energy value of oligosaccharides, the average value of nondigestible oligosaccharides was 2.71 kcal/g (Livesey and Elia, 1988; Miller and Wolin, 1979; Smith and Bryant, 1979; Tokunaga et al., 1989). But, the value should be recalculated as 1.87 kcal/g, as there is only a 69% of apparent utilization efficiency of SCFAs. This value is less than 50% of energy value of sucrose (4 kcal/g) (Tokunaga et al., 1989).

3.2.1.2. Prebiotic Effect

Prebiotics are defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, so
improving host health (Gibson and Roberfroid, 1995). Metabolism of prebiotics follows the same pattern as low calorie food. Prebiotics stimulate beneficial microflora such as the Bifidobacteria and Lactobacilli, and suppress harmful bacteria such as *Clostridium* sp. and *Eubacterium* sp. (Gibson and Wang, 1994; Gibson and Roberfroid, 1995). Beneficial bacteria produce a large amount of SCFAs and cause pH decreases in the lumen of the large intestine (Gibson and Wang, 1994; Gibson and Roberfroid, 1995). They can survive in acidic condition, whereas less desirable bacteria are generally sensitive to the acidic conditions (Gibson and Wang, 1994; Gibson and Roberfroid, 1995). As a result of selective fermentation, the composition of the intestinal microbiota is altered towards a healthier balance (Gibson and Roberfroid, 1995). According to Gibson (2001), prebiotic oligosaccharides produce subordinate health beneficial effects: improved tolerance to lactose due to the presence of lactase, protection from gastroenteritis by the excretion of antimicrobial substances or competition for colonization sites and nutrients, prevention of bowel cancer and coronary heart disease, vitamin B synthesis, and decreased carcinogenesis from digestion contents by reducing enzyme levels for β-glucuronidase, β-glucosidase, nitroreductase and urease in intestinal flora.

### 3.2.1.3. Low Cariogenic Property

Biosynthesis of extracellular water insoluble glucans (mutan) from sucrose facilitates the accumulation of oral streptococci such as *S. mutans*, *S. sobrius*, and *S. sanguis*, and increases the cellular adhesion and serves as a matrix for plaque formation (Michalek and Childers, 1990; Walker, 1972; Guggenheim and Newbrun, 1969). This process results ultimately in dental caries (Lee 2005; Rolla et al., 1983). Some oligosaccharides are useful in preventing dental plaque. Cariogenic oligosaccharides, such as maltooligosylsucrose known as coupling sugar, and palatinose (6-O-α-D-glucosyl-D-fructofranose: isomaltulose) inhibit the production of insoluble glucans and fatty acids because they are not metabolized by the oral streptococci (Ooshima et al., 1983; Otaka, 2006).
3.2.2. Anti-fungal Agents

Antifungal agents are compounds that selectively eliminate fungal pathogens from a host, with minimal toxicity to the host (Dixon and Walsh, 1996). Control of fungi is crucial to prevent losses in food supplies and to decrease the fatal effects of fungal infections in people with weakened immune systems (Groll et al., 1998; Georgopapadakou and Tkacz, 1995). Studies of antifungal agents have lagged behind research on antibacterial agents (Ghannoum and Rice, 1999). Bacteria are prokaryotic and offer numerous structural and metabolic targets that differ from those in human hosts. However, fungi are eukaryotic with the same biochemistry as mammalian cells resulting in many similarities between fungi and host cells in both cell structure and metabolism. Due to these similarities, many antifungal agents can be toxic to host cells as well as fungi. This causes toxic side effects on exposure to antifungal agents. There is a lack of selective toxicity of antifungal agents producing a poor selection of clinically available drugs (Abu-Elteen and Hamad, 2005). Poor solubility of many antifungal agents and poor absorption through the gastrointestinal tract reduce feasibility of oral administration and increase the levels of toxicity associated with the use of antifungal agents (Abu-Elteen and Hamad, 2005). Antifungal agents are usually classified into three major groups by their site of action: antimicrobial agents affecting fungal sterols, compounds inhibiting nucleic acids, and compounds active against fungal cell walls (Ghannoum and Rice, 1999).

3.2.2.1. Currently Used Antifungal Agents

3.2.2.1.1. Antimicrobial Affecting Fungal Sterols

Antifungal agents such as azoles, polyenes, and allylamine/thiocarbamates can inhibit synthesis or interact with ergosterol (Parks and Casey, 1996). Ergosterol is the predominant component of fungal cell membrane and acts as a bio-regulator of membrane integrity controlling membrane fluidity and asymmetry (Nozawa and Morita, 1986).
Azole antifungal agents generally have five-membered rings containing either two or three nitrogen molecules-imidazole and trizoles (Holt, 1980). The heme protein, the primary target of azoles, catalyzes cytochrome P-450-dependent 14α-demethylase. The enzyme controls depletion of ergosterol and accumulation of sterol precursors including 14α-methylated sterols (lanosterol, 4, 14-dimethylzymosterol, and 24-methylenedihydrolanosterol). Blocking this enzyme may result in the formation of a plasma membrane with altered structure and function (Sanati et al., 1997; Hitchcock et al., 1990). However, long-term use of azoles can cause liver toxicity (Parks and Casey, 1996). Moreover, the use of azoles-fluconazole, ketoconazole and miconasol has been reported to be clinically resistant by Candida spp. (Lyman and Walsh, 1992; Sojakova et al., 2004).

Polyene is called a large macrolide ring closed by the formation of an internal ester of lactone (Abu-Elteen and Hamad, 2005). Amphotericin B, nystatin, and flucytosine are widely used polyene antifungal agents. Amphotericin B is considered to be the “gold standard” for antifungal drugs. However, its use is limited due to infusion related reactions and nephrotoxicity (Fanos and Cataldi, 2000). Polyene increases cell membrane permeability, which leads to leakage of cellular constituents (amino acids, sugars and other metabolites), resulting in cell lysis and death. The polyene compounds interact with sterols in cell membranes, ergosterol in fungal cells or cholesterol in human cells to form channels through the membrane that cause the cells to become leaky (Parks and Casey, 1996).

Allylamine inhibits the enzyme squalene epoxidase in the fungal biosynthesis of ergosterol (Katz, 1999), and is used for the treatment of superficial dermatophytic and yeast infections (Perez, 1999).

3.2.2.1.2. Compounds Inhibiting Nucleic Acids

The compound that disrupts nucleic acids is 5-fluorocytosine, a fluorinated pyrimidine. Once 5-fluorocytosine enters fungal cells aided by a permease, it is converted to 5-fluorouracil by cytosine
deaminase. The 5-fluorouracil is subsequently converted by UMP pyrophosphorylase into 5-fluorouridylic acid (FUMP), phosphorylated further and incorporated into RNA, resulting in disruption of protein synthesis (Polak and Scholer, 1975). The 5-fluorouracil is also converted to 5-fluorodeoxuryridine monophosphate, a potent inhibitor of thymidylate synthase, an enzyme involved in DNA synthesis and nuclear division. Therefore, 5-fluorocytosine can interfere with pyrimidine metabolism, as well as RNA, DNA, and protein synthesis in fungal cells (Diasio and Myers, 1978).

3.2.2.1.3. Compounds Active against Fungal Cell Walls

The fungal cell wall is a multilayered structure composed of chitin, β-glucan and mannan, which is not replicated elsewhere in nature. The outer layers are composed of mannan, mannoprotein, β-(1,6)-glucan, whereas the inner layers are predominantly β-(1,3)-glucan and chitin with some mannoprotein (Hector, 1993).

3.2.2.1.3.1. Chitin Synthesis Inhibitors

Chitin is a long linear homopolymer of β-1,4-linked N-acetylglucosamine which constitutes 1-2 % of the yeast cell wall and 10-20% of the cell walls of filamentous fungi, Neurospora and Aspergillus, by dry weight (Bowman and Free, 2006; De Nobel et al., 2000; Klis et al., 2002). Despite being a minor component in the fungal cell wall, its structure is pivotal to the overall integrity of the cell wall and its enormous tensile strength (Bowman and Free, 2006). Without chitin, the cell wall becomes disordered, mal-formed and osmotically unstable, leading to cell death (Bago et al., 1996; Specht et al., 1996). Chitin synthase catalyzes the transfer of N-acetyl glucosamine from uridine diphosphate (UDP)-N-acetylglucosamine to a growing chitin chain (Bowman and Free, 2006). At sites of active growth and cell wall remodeling, hydrogen bondings between the newly formed chitin polymers form chitin microfibrils and subsequently crystallize chitin in the extracellular space immediately adjacent to the plasma membrane. In yeast cells, this process occurs at the bud tips during polarized growth and the bud
neck during cytokinesis. For filamentous fungi, cell wall synthesis localizes at the hyphal apex or the growing tips (Bowman and Free, 2006). The best chitin synthesis inhibitors are nikkomycins, demethylallosamidin, and polyxins (Bowman and Free, 2006; Abu-Elteen and Hamad, 2005). These agents function as mimics or decoys of the enzyme substrate UDP-N-acetylglucosamine for chitin synthase (Abu-Elteen and Hamad, 2006; Hector, 1993). However, their effectiveness to control mycoses has not been proved. Combined treatments using nikkomycins and fluconazole and itraconazole have often been used (Abu-Elteen and Hamad, 2006; Bowman and Free, 2006).

3.2.2.1.3.2. Glucan Synthesis Inhibitors

The fungal cell wall contains approximately 50-60% glucans. A β-(1,3)-glucan is the major structural constituent (65-90%) in the fungal cell wall (Bernard and Latge, 2001; Klis et al., 2001). The cell walls of *S. cerevisiae* and *C. albicans* contain branched β-(1,3)- and β-(1,6)-glucans (Cabib et al., 1988) whereas the cell walls of many filamentous fungi including *Neurospora crassa* and *A. fumigates* contain only β-(1,3)-glucan (Borkovich et al. 2004; Fontaine et al., 2000).

The multisubunit enzyme, β-1,3-D-glucan synthase (1,3-β-D-glucan 3-β-D-glucosyltransferase; EC 2.4.1.34; GS) catalyzes the formation of long linear chains of glucans connected through β-1,3-linkages from UDP-glucose (UDPG) (Cabib et al., 1988). A genetic study of β-1,3-D-glucan synthase in *S. cerevisiae* identified that the gene contains two functionally redundant catalytic subunits-*FKSI* and *FKS2* and one regulatory protein-*RHO1* (Douglas et al., 1994; Mazur et al., 1995; Qadota et al., 1996). Fks1p is the major protein responsible for GS activity during vegetative growth whereas Fks2p appears to be important for sporulation (Marzur et al., 1995). The activation and the movement of Fks1p to the plasma membrane are required for cell wall β-1,3 glucan synthesis and have been shown in many fungi including *Schizosaccharomyces pombe* and *Aspergillus* species (Lesage and Bussey, 2006). When *FKSI* is disrupted, the strains are viable but decrease the β-1,3-D-glucan synthase activity by 5-8 fold and are
three fold more sensitive to pneumocandin (Douglas et al., 1994). Disruption of \textit{FKS2} is not lethal. However, simultaneous deletion of \textit{FKS1} and \textit{FKS2} is lethal, suggesting that they encode interchangeable subunits of β-1,3-D-glucan synthase with common functions (Douglas et al., 1994; Mazur et al., 1995). \textit{S. cerevisiae RHO1} gene is essential for survival. The \textit{FKS} and \textit{RHO1} genes are also highly conserved among fungi and required for cell viability (Bowman and Free, 2006; Beauvais et al., 2001).

The β-glucan synthase inhibitors, especially echinocandins, act as specific noncompetitive inhibitors of β-(1,3)-glucan synthase (Hector, 1993) without affecting nucleic acid or mannan synthesis. These inhibitors also have secondary effects on other components of intact cells including a reduction in the ergosterol and lanosterol and an increase in the chitin in the cell (Pfaller, 1989). Use of capsofungin, the first clinically used echinocandin, and other echinocandins produce changes in filamentous fungal growth, morphology and cell wall structure of the hyphae (Kurtz et al., 1994) since the \textit{FKS} genes localize at the hyphal apex (Beauvais et al., 2001; Douglas et al., 1994). This is supported by observations that a 1,3-β-D-glucan-specific fluorochrome stained cell has the most intense fluorescence at the hyphal apex and a new cell wall was formed exclusively at the hyphal apices in \textit{A. nidulans} (Momany et al., 1999). Therefore, inhibition of β-(1,3)-glucan synthase causes cytological and ultrastructural changes in fungi characterized by growth of pseudohyphae, swollen hyphae, thickened cell wall, or buds failing to separate from mother cells. Cells become osmotically sensitive to lysis which is restricted largely to the growing tips of budding cells (Bozzola et al., 1984; Cassone et al., 1981).

\subsection*{3.2.2.2. Other Potential Antifungal Agents}

Danac et al. (2007) attempted to inhibit chitin biosynthesis using selectively functionalized β(1→4)\textit{N}-acetylglucosamine (GlcNAc) at C-4. According to the hypothesis of Danac et al. (2007), a
chain terminator, GlcNAc residue with modified 4-hydroxyl might lead to a chitin chain-termination step since the required 4-hydroxyl, at which subsequent units would had been added, would then be absent. They found that GlcNAc-derived ester and oxazoline having N$_3$ at C4 interfered with adhesion and germination of the dermatophyte, *Trichophyton rubrum* (Danac et al., 2007).

According to Bisogno et al. (2007), cinnamic acid derivatives, more specifically (E)-3-4-[methoxy-3-(3-methylbut-2-enyl) phenyl] acrylic acid exhibited antifungal activity against *A. niger*, *A. flavus*, and *A. terreus*. They also investigated the relationship between the structure of cinnamic acid derivatives and antifungal activity. The absence of the double bond of the side chain in cinnamic acid derivatives did not affect fungal growth. They concluded that the presence of the double bond confers a particular conformational and electronic characteristic to these compounds. A COOH group in the side chain of these compounds seemed to be necessary, but not by itself sufficient to produce antifungal potency.

A peptide, Ay-AMP, isolated from *Amaranthus hypochondriacus* seeds has a chitin-binding property and inhibited the fungal growth in *C. albicans*, *Trichoderma* sp., *Fusarium solani*, *Penicillium chrysogenum*, *Geotrichum candidum*, *A. candidus*, *A. schraceus*, and *Alternaria alternate* (Rivillas-Acevedo and Soriano-Garcia, 2007). This peptide settled along the fungal cell walls and accumulated at septa and hyphal tips by the union to the fungus cell-wall chitin.

Zumbuehl et al. (2007) found that amphogel, a dextran-based hydrogel into which amphotericin B (AmB) is absorbed, killed *C. albicans* within two hours of inoculation and it effectiveness was maintained for at least 53 days. The dextran could be a protein repellent (Frazie et al., 2000) and mitigate biofilm formation (Verstrepen et al., 2004), and gave AmB a hydrophilic matrix (Zumbuehl et al., 2007). This hydrogel was biocompatible and did not cause hemolysis in human blood.

The steroid saponins have been reported as antifungal agents in many literatures (Zhang et al.,
2006; Ekabo et al., 1996; Mshvildadze et al., 2000; Renault et al., 2003; Sautour et al., 2004). Zhang et al. (2006) concluded a spirostanol framework with a number of oligosaccharide residue attached at the C-3 of the aglycon might closely relate to the antifungal efficacy of steroid saponins. However, a steroid saponin without a carbonyl base at C-12 (Zhang et al., 2006) and furostanol-type steroidal glycoside (Hufford et al., 1988) did not have antifungal activity.

Lactic acid bacteria metabolites such as organic acids, fatty acids, and cyclic dipeptide have been exploited as antifungal agents (Valerio et al., 2004; Magnusson et al., 2003; Lavermicocca et al., 2000). Phenyllactic and 4-hydroxy-phenyllactic acids effectively inhibited the growth of A. niger, A. flavus, Eurotium rubrum, E. repens, E. fibuliger, Penicillium corylophilum, P. roqueforti, and Monilia sitophila (Valerio et al., 2004; Lavermicocca et al., 2000). Some cyclo peptides: cyclo(Phe-t-4kOH-t-Pro), cyclo(Phe-trans-t-4kOH-t-Pro), and cyclo (Phe-Pro), and cyclo(Phe-4kOH-Pro) isolated from lactic acid bacteria showed antifungal activity (Ström et al., 2002; Magnusson et al., 2003). According to Cabo et al. (2002), the synergism between lactic acid produced from lactic acid bacteria and acetic acid from the MRS growth medium showed strong antifungal activity. However, Magnusson et al. (2003) negated the possibility that the degree of fungal inhibition related to production of lactic or acetic acid as changes in organic acid concentrations did not explain the varying degrees of inhibition of fungi. They suggested that lactic acid bacteria from different genera and species, even different environments can exhibit antifungal activity producing several different compounds and proposed more study on compounds and action modes.

We have verified the potential of cellobio-oligosaccharides as an anticariogenic agent and an α-glucosidase inhibitor. It is possible that these cellobio-oligosaccharides may be a potential antifungal agent, which correlates with 1,3-β-D-glucan synthase inhibition.
3.3. MATERIALS AND METHODS

3.3.1. Cariogenicity

3.3.1.1. Microorganisms and Isolation of Mutansucrase

Oral bacteria used were collected by a cotton swab from teeth and streaked onto a brain heart infusion (BHI) agar containing 4% sucrose. They were grown at 37°C until visible colonies of *Streptococcus mutans* and *S. sorbrinus* appeared. The colonies were grown in 1 L BHI at 37°C with shaking at 150 rpm for 24-36 hours to produce mutansucrase. After fermentation, the culture was harvested, centrifuged and concentrated to 100 ml using a 30 K cut-off membrane filter. One unit of mutansucrase was defined as the amount of enzyme that catalyzes the formation of 1 µmol of fructose per minute at 37°C and pH 7.0 from 100 mM sucrose.

3.3.1.2. Inhibition of Mutansucrase and Insoluble Glucan Synthesis

The inhibition of CBO on the synthesis of water insoluble glucans by oral *Streptococcus* species and mutansucrase was determined. *Streptococcus* species were inoculated in 2x BHI broth containing 1M sucrose and 50 mM CBO and were cultured in glass vials at 37°C for 48 hours. Mutansucrase was incubated in 20 mM HEPES (pH 7.0) containing 1 M sucrose and 50 mM CBO in glass vials at 37°C for 48 hours. The supernatants of individual reaction mixtures were discarded and insoluble glucans remained in the vial. For comparison of the amount of insoluble glucans produced, the synthesized glucans were washed with a 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES; pH 7.0) and dissolved in 0.5 N NaOH. The absorbance of water insoluble glucans was measured at 550 nm (Lee et al., 2003). For visualization, synthesized glucans were dyed with a drop of dental disclosing solution.

3.3.2. α-Glucosidase Assay

Enzyme reaction mixtures contained 4.9 U α-glucosidase, 88 mM maltose, and different concentrations of cellobio-oligosaccharides (0-3.3 mM) in 20 mM HEPES buffer (pH 7.0) and were
incubated for 90 minutes at 25°C. α-Glucosidase (maltase) activity was assayed according to the method of glucose oxidase-peroxidase using a glucose assay kit (Sigma-Aldrich, St. Louis). Glucose was used as a standard curve. The color developed final product (oxidized o-dianisidine) was measured at 540 nm.

3.3.3. Antifungal Effect

3.3.3.1. Microorganism and Culture Condition

*A. terreus* was maintained on potato dextrose agar (PDA) medium for 5 to 7 days at 28°C. Conidia were collected with a cotton swab and suspended in 0.9% NaCl solution with 0.05% Tween 20. The heavy particles were allowed to settle for 2 hours in cold (4°C) solution. For the effect of CBO on the growth of *A. terreus*, 2.5 x 10^4 conidia were inoculated into 2.9 ml of potato dextrose broth (PDB) with CBO and incubated for 10 days at 28°C.

3.3.3.2. Preparation of (1,3)-β-D-Glucan Synthase

For glucansynthase production, 4.5 x 10^8 conidia were inoculated into 500 ml of YME medium containing 0.4% yeast extract, 1.0% malt extract, and 0.4% dextrose and incubated at room temperature for 1 to 2 days with shaking at 150 rpm. The spherical mycelia grown on YME medium with shaking were harvested by centrifugation at 1,500 x g for 10 minutes. Cells were washed extensively with water and then centrifuged at 1,500 x g for 10 minutes. Cell breakage was performed using 20 cycles (1 min each) of vortexing with prechilled glass beads in chilled extraction buffer containing 50 mM HEPES (pH 7.2), 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol, 1 µg of leupeptin per ml, and 10 µM GTPrS at approximately 5 ml of buffer per cell (g, wet weight). Cells were cooled for 5 minutes on ice between cycles. The homogenate was centrifuged at 1,500 x g for 10 minutes to remove cell debris. After centrifugation at 23,000 x g for 10 minutes to remove mitochondrial membranes, the supernatant was ultra-centrifuged at 100,000 x g for 1 hour to recover microsomal membranes in a pellet. This pellet was resuspended in one-tenth the original volume of cold storage buffer containing 50 mM HEPES (pH
7.2), 1 mM EDTA, 1 mM DTT, and 20% glycerol. Protein concentration of isolated (1,3)-β-glucan synthase was 2.5 mg/ml. All procedures for enzyme preparation were carried out at 4°C. Protein concentration was determined by the method of Bradford with Bio-Rad reagent and bovine serum albumin was used as standard.

3.3.3.3. Assay of (1,3)-β-D-Glucan Synthase

Glucan synthase activity was determined by the modification of a fluorescence method (Ko and Cheng, 2005; Shedletzky et al., 1997). The assay mixture (150 µl) contained 27 mM HEPES (pH 7.2), 7 µM GTP, 1.3 mM EDTA, 0.17% Brij 35, 2.2% glycerol, 0.7 mM UDP-Glc, and isolated GS enzyme (0.83 µg/µl). For inhibition studies, 0.12-0.63 µg of CBO were added to the desired mixture. Reactions were started by addition of GS, incubated at 22°C for 105 minutes, and terminated by an addition of 10 µl of 6 N NaOH. Glucans produced were solubilized in a water bath at 80°C for 30 minutes followed by addition of 20 µl of one forth diluted Sirofluor™ (Biosupplies, Australia). The mixtures were incubated for 50 minutes at 22°C and measured with a fluorescence spectrophotometer (FluoroLog) at an excitation wavelength of 390 nm and an emission wavelength of 455 nm. Standard curves were constructed using various concentrations of yeast glucan, dissolved in 300 µl of 1 N NaOH by heating 30 minutes at 80°C, containing the same components as the reaction mixtures except enzyme.

3.3.3.4. Electron Microscopy

A study of the morphologic aspects was conducted by scanning electron microscopy (SEM; Nishiyama et al, 2005). Conidia (3.0 x 10⁴) were inoculated in PDB and incubated at 28°C. After 16 hour incubation, CBO was added to a test tube and H₂O into a second tube as a control. They were further incubated for two days at 28°C. For SEM, cultures were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C for 2 hours. After being washed with the buffer, specimens were post-fixed for 2 hours with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) at 4°C. Samples were
dehydrated in graded acetone, freeze-dried in \( t \)-butyl alcohol and sputter–coated with palladium-gold. Observation was carried out with a scanning electron microscope (Nishiyama et al., 2001).

3.4. RESULTS AND DISCUSSION

3.4.1. Cariogenicity

The CBO inhibited effectively the synthesis of water insoluble glucans in the presence of sucrose by mutansucrase (Figure 3.1). In the presence of 50 mM CBO and 1 M sucrose, only 4% of insoluble glucans were produced compared to that of 1 M sucrose reaction mixture. Cellobiose (50 mM) did not affect insoluble glucan formation as almost same amount of insoluble glucans was produced as in the control. Insoluble glucans in solution were swirled along the inner layer of a glass vial, and the liquid discarded. Then the carbohydrates were dyed with a dental disclosing solution. The insoluble glucans adhered as an inner layer on a glass vial as dental plaque does on teeth (Figure 3.2). The quantity of insoluble glucans adhered to a glass was less for the CBO mixture than the control or cellobiose test. The inhibitory effect of CBO against mutansucrase might be caused by an acceptor reaction of glucosyltransferase, leading to termination of glucan synthesis from sucrose. Our data are consistent to that observed with isomaltosylfructoside (Nisizawa et al., 1986), fructosylxyloside (Takeda and Kinosh, 1995), maltosylsucrose (Lee et al., 2003), glucooligosaccharides and fructooligosaccharides (Nam et al., 2007). Dental caries is mostly caused by \( S. \) mutans and \( S. \) sobrinus which synthesize extracellular water insoluble glucans from sucrose by glucosyl transferase (Hamada and Slade, 1980). The insoluble glucans become plaque on teeth and result in tooth decay. In addition, \( S. \) mutans and \( S. \) sobrinus synthesize intracellular polysaccharides as carbohydrate reserves, which can be converted to acids when dietary carbohydrates are available (Marsh, 1999). CBO has a potential to become an active ingredient in dental care products since it inhibits glucan formation.
Figure 3.1. Effect of addition of cellobiose or CBO on the formation of insoluble glucan by *mutansucrase*. Relative amount of insoluble glucans is the percent of the amount of insoluble glucans in the test to that in the control mixture. Error bars show the standard deviation errors of the mean.
Figure 3.2. **Insoluble glucans synthesized under different conditions in glass vials.** Panel A, before addition of disclosing solutions; Panel B and C, after addition of disclosing solutions. Insoluble glucans were synthesized for 48 hours at 37 °C by mutansucrase in the presence of 1 M sucrose and 20 mM HEPES (for a control), 50 mM cellobiose (for a cellobiose), or 50 mM CBO (for a CBO).
3.4.2. α-Glucosidase Inhibition

The inhibitory activity of CBO against α-glucosidase (Figure 3.3) was determined. Increased concentrations of CBO decreased the amount of glucose produced from maltose by α-glucosidase.

α-Glucosidase activity was 86% of control in the presence of 0.7 mM CBO, 77% in the presence of 2.0 mM CBO, and 55% in the presence of 3.3 mM CBO. Inhibition of α-glucosidase can delay carbohydrate digestion and glucose absorption, attenuating postprandial hyperglycemia (Bischoff, 1995; Lee, 2005; Truscheit et al., 1981). At present, α-glucosidase inhibitors such as acarbose, miglitol, and voglibose are commonly used to reduce the postprandial hyperglycemia by interfering with the digestion of dietary carbohydrates (Moordian and Thurman, 1999; Williamson et al., 1992). Acarbose is widely used as a therapeutic agent for the treatment of patients with type II diabetes mellitus (Chiasson et al., 2002; Balfour, 1993). Concern about its side effects is driving the search for alternative safe and efficient α-glucosidase inhibitors (Matsuura et al., 2002).

3.4.3. Antifungal Effect

3.4.3.1. Inhibition against Glucan Synthase

Antifungal agents, the GS inhibitors have been validated as an effective treatment of fungal infections because these agents inhibit fungal cell wall synthesis, a target unique to lower eukaryotes (Onishi et al, 2000). The effect of CBO on GS, the essential enzyme that forms β-(1,3)-glucan fibrils from UDP-glucose, was evaluated. Inhibition was largely dose dependent (Figure 3.4). The concentration of 0.12 g/ml CBO was not sufficient to inhibit GS activity. The 50% inhibitory concentration (IC$_{50}$) for CBO was 0.36 g/ml.

The role of CBO on GS was further evaluated by a kinetic study over a range of concentrations of UDP-glucose ranging from 0.05 to 8 mM with CBO added at concentrations of 0, 0.24, and 0.36 g/ml. The reaction velocity was calculated, and the Lineweaver-Burk plot of 1/[substrate] and 1/velocity at
Figure 3.3. Inhibition effect of CBO against α-glucosidase. The reaction mixture contained 4.9 U α-glucosidase, 88 mM maltose, and different concentration of cellbio-oligosaccharides (0, 0.7, 2.0, and 3.3 mM) in 20 mM HEPES buffer and incubated for 90 min at 25°C. Error bars show the standard deviation errors of the mean.
Figure 3.4. Inhibition of glucan synthase activity by cellbio-oligosaccharides. Relative activity is the percent of 1,3-β-D-glucan synthase activity (GS) at test concentration of cellbio-oligosaccharides (CBO) to the GS activity at none CBO added reaction mixture (control). For the determination of GS activity, 0.7 mM UDP-G was reacted with 0.83 µg/µl GS in 27 mM HEPES (pH 7.2) containing 7 µM GTP, 1.3 mM EDTA, 0.17% Brij 35, and 2.2% glycerol in the addition of 0, 0.12, 0.24, 0.36, and 0.48 g/ml CBO at 22°C for 105 min. A Sirofluor™ binding with 1,3-β-D-glucans was then conducted as described in the material and method section. The fluorescence was measured excitation wavelength of 390 nm and emission wavelength of 455 nm. Error bars show the standard deviation error of the mean.
three oligosaccharide concentrations are illustrated in Figure 3.5. The non-parallel lines which converge at $x<0$ and $y>0$ are consistent with mixed type of inhibition.

There are no reports on the relationship of oligosaccharides containing mixed $\beta(1\rightarrow4)$, $\alpha(1\rightarrow2)$, and $\alpha(1\rightarrow6)$ linkages and fungal glucan synthases. A cellotriose, comprising glucose linked with only $\beta-1,4$, enhanced glucan synthase isolated from *Euglena gracilis* (Marechal and Goldemberg, 1964). Differences between our CBO and this cellotriose (Marechal and Goldemberg, 1964) demonstrate that the type of linkages may be important in altering glucan synthase activity. Cellobiose has been reported to be a stimulator for glucan synthase production in sugar beet (Morrow and Lucas, 1986) and *Euglena gracilis* (Marechal and Goldemberg, 1964). However, cellobiose does not stimulate the glucan synthase of *S. cerevisiae* (Lopez-Romero and Ruiz-Herrera, 1978) and the germinating peanut, *Arachis hypogaea* (Kamat et al., 1992). A very simple sugar based chemical, $\delta$-gluconolactone was an effective inhibitor of (1→3)-$\beta$-D-glucan synthase in the sugar beet (Morrow and Lucas, 1987) and in *S. cerevisiae* (Lopez-Romero and Ruiz-Herrera, 1978).

So far, most GS inhibitors have been categorized in three chemical classes of compounds: lipopeptides comprising cyclic hexapeptides $N$-linked to a fatty acyl side chain, papulacandins consisting of a modified disaccharide linked to two fatty acyl chains, and acidic terpenoids (Douglas, 2001; Onishi et al., 2000; Tracz 1992; Traxler et al., 1977).

### 3.4.3.2. Morphological Changes in *Aspergillus terreus*

Most glucan synthase inhibitors induce profound morphological changes in fungal hyphae which correlate with inhibition of glucan synthase (Kurtz et al., 1994; Bozzola et al., 1984; Cassone et al., 1981). Observation of hyphal changes after addition of CBO was conducted using SEM. Hyphae of *A. terreus* showed distinct structural differences between control and CBO treated cultures (Figure 3.6). The bud scar rings are found in several hyphal tips on the control but none on CBO treated *A. terreus*. 
Figure 3.5. Lineweaver-Burk plot: inhibition of (1→3)-β-D-glucan synthase by celllobio-oligosaccharide. The assay mixtures contained 27 mM HEPES (pH 7.2), 7 µM GTP, 1.3 mM EDTA, 0.17% Brij 35, and 2.2% glycerol, varying concentration of UDP-G (0.05, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 µg/µl) also containing 0.83 µg/µl 1,3-β-D-glucan synthase (GS). The reaction allowed to react for 105 min at 22°C. GS activity was also measured in the absence or presence of 0, 0.24, and 0.36 g/ml celllobio-oligosaccharides. A Sirofluor™ binding with 1,3-β-D-glucans was then conducted as described in the material and method section. The fluorescence was measured excitation wavelength of 390 nm and emission wavelength of 455 nm.
CBO treated *A. terreus* failed to bud their cells, and the population did not increase. In addition, their widths of hyphae were different between the two cultures when 20 hyphae were randomly selected and measured. The average width (3.4 µm) of twenty hyphae in CBO treated *A. terreus* was 1.35 fold larger than that (2.5 µm) in the control. In the presence of CBO, the cells grew with swollen hyphae, indicating inhibition of glucan synthesis (Kurtz et al., 1994). This observation supports the fact that glucan synthesis inhibition produces stunted, swollen hyphae, caused from a weakened cell wall that expands under high internal pressure (Onishi et al., 2000). In the work of Kurtz et al. (1994), pneumocandin treated *A. fumigates* caused swelling and distension of the hyphae. Although the abundance of 1,3-β-D-glucans in the cell walls formed during different stages of the *A. fumigatus* life cycle is not well characterized, the focus of new cell wall synthesis is the hyphae during vegetative growth (Archer, 1977; Beauvais et al., 2001; Ruiz-Herrera, 1992), and inhibition of 1,3-β-D-glucan synthesis has profound effects on cell wall structure in *A. fumigatus* (Kurtz et al., 1994). Inhibition of glucan synthesis results in structural changes, characterized as pseudohyphae, swollen hyphae, thickened cell wall, or buds failing to separate from mother cells (Kurtz et al., 1994; Bozzola et al., 1984; Cassone et al., 1981). We explored the effect of CBO on *A. terreus*, grown in PDB and PDA during extended incubation up to ten days at 28°C (Figure 3.7). When *A. terreus* was grown in PDB medium, it formed tangled hyphal masses on the surface in a tube. However, they were not observed when *A. terreus* was incubated with CBO in PDB medium. There was substantial growth in the untreated culture during the course of the experiment.

There is no comparable data that oligosaccharide alone works as an antifungal. Almost all proposed antifungal agents have complicated structures. Nevertheless, Kaur et al. (2006) suggested that a small and simple sugar acid, D-gluconic acid from *Pseudomonas* strain AN5 may have antifungal activities against take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici*. Some researchers have been reported that cellobiose-based lipids have fungicidal activities. Complex
Figure 3.6. Scanning Electron Microscopy (SEM) images of *A. terreus* cells. Panels: A, control growth of cells (no cellbio-oligosaccharide); B, growth of cells treated with cellbio-oligosaccharides. Arrows point at sporulation of the cells. Bars represent 20 µm at 1.34 kX magnification.
Figure 3.7. In vitro growth of *A. terreus* in PDB and PDA. Panels: A, control growth of cells; B, growth of cells treated with celllobio-oligosaccharides. All tubes and petri-dishes were photographed ten days after incubation at 30°C.
celllobiose-lipids of yeast fungi *Cryptococcus humicola* and *Pseudozyma fusiformata* (ustilagic acid B) inhibited the growth of a number of species important for medicine: *Candida. albicans*, *C. glabrata*, *C. viswanathii*, *F. neoformans*, and *Clavispora lusitaniae* (Kulakovskaya et al., 2007; Kulakovskaya et al., 2006). They may stimulate the release of ATP from the test culture cells, indicating an increase the permeability of the plasma membrane, and resulting in cell death (Puchkov et al., 2001; Kulakovskaya et al., 2004). Mimee et al. (2005) isolated Flocculosin, a low molecular weight celllobiose-lipid, from the yeast like fungus *Pseudozyma flocculosa* to investigate antifungal activity. Flocculosin significantly inhibited the growth of *Candida lusitaniae*, *C. neoformans*, *Trichosporon asahii*, and *C. albicans*. Synergistic activity was also verified between flocculosin and amphotericin B, suggesting the potential for amphotericin B having much lower MIC. Most isolated celllobiose-lipids have considerable efficacy as potential antifungal agents under acidic conditions (Kulakovskaya et al., 2007; Mimee et al., 2005).

Based on our data, CBO has a great potential to function as a new class of antifungal agent against fungi which correlate with 1,3-β-D-glucan synthase inhibition.

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CHAPTER 4. CONCLUSIONS

The utilization of cellulose has attracted much attention because of its potential as a feedstock for bioethanol production. The economics of bioethanol production requires large volumes of product at a low cost. For a “biorefinery” to be economically viable, it should produce a range of products, not just bioethanol. Current research has focused on biofuel production and a potentially valuable product, cellobiose has been overlooked. This research has focused on a developing technologie(s) where lignocellulose conversion can be modified such that cellobiose and other oligomers are produced as products. The inhibition of cellulase by glucose degradation products-gluconolactone and gluconic acid has been previously reported (Holtzapple et al 1990; Philippidis et al 1993). However, thought has not been given to use cellulase inhibition to increase cellobiose concentrations. The inhibitors-glucose oxidase, gluconic acid, and gluconolactone inhibited selectively the β-glucosidase associated with the cellulase “complex”, since they produced equivalent (in the presence of glucose oxidase and gluconolactone) or similar (in gluconic acid) inhibition on either pure β-glucosidase or β-glucosidase in the cellulase “complex”. Our inhibition studies agree with others (Dixon et al., 1979; Holtzapple et al., 1990) in that both gluconolactone and gluconic acid exhibit mixed inhibition. Box-Behnken data demonstrated that cellobiose production was largely dependent on the concentrations and combination of cellulose as a substrate, cellulase, inhibitor, and reaction time as well as the type of inhibitor. Ammonia treated sugarcane bagasse used as a substrate in these reactions, produced cellobiose in the same manner as pure cellulose, although cellobiose yields from ammonia treated sugarcane bagasse were lower than with pure cellulose, probably due to the presence of “undesired” components in the hydrolysis system. Mixtures of cellulase and any of the test inhibitors produced between 7.4-10.4 times more cellobiose than cellulase in the absence of inhibitor(s). Gluconolactone was the most effective β-glucosidase inhibitor for producing 31.2% cellobiose of complete conversion of cellulose to cellobiose.
The yield of cellobiose was 23.7% with glucose oxidase, similar to 21.9% with an oxidized product of glucose, gluconic acid. Cellobiose produced using this cellulase modification system is significantly higher than has been previously achieved, yields between 0.07 and 7.5% (Homma et al., 1993; Tanaka, 2000; Tanaka and Oi, 1985). If there is a market for cellobiose, significant quantities could be produced by this method to produce an additional product from a biorefinery.

Dextranucrase from *Leuconostoc mesenteroides* B-512FMCM was used to synthesize, by transglycosylation, cellobio-oligosaccharides from sucrose and cellobiose. Enzymatic transglycosylation for oligosaccharide synthesis has advantages in time-, cost-, and process step-effectiveness over either a Leloir synthesis which requires sugar nucleotides as a glucosyl donor or chemical methods (Nahalka et al., 2003). Dextranucrase was produced free of dextran and sucrose phosphorylase which could affect transglycosylation efficiency. The reaction for transglycosylation of sucrose onto cellobiose to produce oligosaccharides was conducted at a pH of 5.2 and a temperature of 30˚C. The concentration of dextranucrase was 54 U, the concentration of sucrose was 289 mM and the concentration of cellobiose was 250 mM. The cellobio-oligosaccharide yield reached 20% based on initial cellobiose concentrations. This process required few steps to obtain final products. They are the transglycosylation reaction of cellobiose and sucrose; dextran removal by ethanol precipitation; concentration; and chromatographic purification of product. The synthesized cellobio-oligosaccharides ranged in size from DP 3 to 6. The major products were trisaccharides. Their proposed structures are α-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-D-glucopyranose and α-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-D-glucopyranose.

These cellobio-oligosaccharides were found to inhibit synthesis of the water-insoluble glucans that are related to dental caries occurrence and the α-glucosidase activity involved in the final step of carbohydrate digestion. This potential anticariogenic function of cellobio-oligosaccharides could be used
in conjunction with dietary sweeteners and soft drinks in the food industry and toothpaste and related products in pharmaceutical industries. The finding that cellbio-oligosaccharides are potential inhibitors of α-glucosidase suggests utilization as dietary supplements or drugs for diabetes mellitus and obesity. In addition to these functions, an *in vitro* β-1,3-D-glucan synthase test and a cell morphology phenomenon indicate the inhibition effects of cellbio-oligosaccharides on fungal cells. The cellbio-oligosaccharides inhibited β-1,3-D-glucan synthase as a mixed type of inhibition. Moreover, cellbio-oligosaccharides treated *A. terreus* grew with swollen hyphae. They have supported the idea that cellbio-oligosaccharides inhibit the growth of *Aspergillus terreus* (and possibly other fungi imperfecti) by inhibiting glucan synthesis. They could be the foundation for a new group of antifungal agents, which affects a fungal cell wall, is safe, consisting of simple sugar structures, with hydrophilic properties.

These observations should be further explored as to the potential commercial value of cellbio-oligosaccharides as fungal inhibitors, as well as additional products to support biofuel production from lignocelluloses.

### 4.1. REFERENCES


APPENDIX

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